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OFFICIAL

PATENT Docket No.: 1038-1160

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Loosmore, et al.
Application No.:		09/857,843
Filing Date	:	September 17, 2001
Title	:	Multi-Component Vaccine Comprising At Least Two Antigens From Haemophilus influenzae To Protect Against Disease
Grp./AU	:	1645
Examiner	:	Jana A. Hines

November 26, 2003

APPEAL BRIEF AND REQUEST FOR EXTENSION OF TIME
TOTAL PAGES 75

BY FACSIMILE **703-872-9307**

The Commissioner of Patents and Trademarks
BOX AF,
Washington, D.C.
20231, U.S.A.

11/28/2003 GWORDOF1 00000042 09857843

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02 FC:1999 320.00 OP

Dear Sir:

Introduction

This Appeal Brief is submitted pursuant to applicant's appeal from a Final Rejection of claims 1-5, 25 and 27 dated December 31, 2002. A Notice of Appeal was filed on June 3, 2003. The enclosed Credit Card Payment Form includes the prescribed fee. In the event of underpayment or overpayment please apply any additional charges or refunds to USPTO Deposit Account Number 500715. Three copies of the Appeal Brief are provided herewith.

Extension of Time

Petition is hereby made under the provisions of 37 CFR 1.136(a) for an extension of four months of the period for filing this Appeal Brief. The enclosed Credit Card Payment

Form includes the prescribed fee. In the event of underpayment or overpayment please apply any additional charges or refunds to USPTO Deposit Account Number 500715.

(1) Real Party of Interest

The real party of interest with respect to this patent application is Aventis Pasteur Limited. Assignments from the inventors to Aventis Pasteur Limited are recorded at Reel 013317/0711, 0724 and 0733 on September 20, 2002.

(2) Related Appeals and Interferences

The appellants, the appellants' legal representatives and assignee, are unaware of any pending appeals or interferences which will directly affect or be affected by or have a bearing on the Board's decision in the pending appeal.

(3) Status of Claims

This application was filed with claims 1-26. In the response dated September 25, 2002 to the Office Action of March 27, 2002 claims 6-24 and 26 were cancelled, claim 25 amended, and new claim 27 added.

Claims 1-5, 25 and 27 were finally rejected in an Office Action dated December 31, 2002. Claims 1-5, 25 and 27 are pending and the subject of this appeal and appear in Appendix I hereto.

(4) Status of Amendments

This application was filed with claims 1-26. Claims 1-5, 25 and 27 are pending and no amendments were filed subsequent to this final rejection.

(5) Summary of Invention

The present invention is directed to an immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae,* including otitis media. The composition comprises at least two different antigens of *Haemophilus influenzae,* at least one of which antigens is an adhesin (claim 1) and wherein said adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae,* (claim 2),

particularly an HMW 1 or HMW 2 protein of the non-typeable strain (claim 3), and said antigen which is not an adhesin is a non-proteolytic heat shock protein of a strain of Haemophilus influenzae (claim 4) and wherein the heat shock protein is an analog of *Haemophilus influenzae* Hin47 protein having a protease activity which is less than about 10% of that of the natural Hin47 protein (claim 5). The invention is further directed to compositions where the HMW protein is recombinantly produced and said antigen which is not an adhesin is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of the natural Hin47 protein in which the Histidine at amino acid position 91 is replaced by Alanine (claim 27). The present invention is further directed to a method of immunizing a host against disease caused by infection with *H. influenzae* (claim 25).

(6) **Issues**

The issues for consideration is the rejection of, claims 1-5 and 25 under 35 U.S.C. 112 1st paragraph and claims 1-5, 25 and 27 under U.S.C. 103(a) as being unpatentable over Barenkamp et al in view of Loosmore et al.

(7) **Grouping of Claims**

All claims do not stand or fall together, but rather each claim is individually patentable.

(8) **Argument**

(a) Background to the Invention

Haemophilus influenzae is the cause of several serious human diseases, such as meningitis, epiglottitis, septicemia and otitis media. There are six serotypes of *H. influenzae,* designated a to f, that are identified by their capsular polysaccharide. *H. influenzae* type b (Hib) was a major cause of bacterial meningitis until the introduction of several Hib conjugate vaccines in the 1980's. Vaccines based upon *H. influenzae* type b capsular polysaccharide conjugated to diphtheria toxoid, tetanus toxoid, or *Neisseria meningitidis* outer membrane protein have been effective in reducing *H. influenzae* type b-induced meningitis. The other serotypes of *H. influenzae* are associated with invasive disease at low frequencies, although there appears to be an increase in the incidence of disease caused by these strains as the incidence of Hib disease

declines. Non-encapsulated or non-typeable *H. influenzae* (NTHi) are also responsible for a wid range of human diseases including otitis media, epiglottitis, pneumonia and tracheobronchitis. The incidence of NTHi induced disease has not been affected by the introduction of the Hib vaccines.

Otitis media is the most common illness of early childhood, with 60 to 70% of all children, of less than 2 years of age, experiencing between one and three ear infections. Chronic otitis media is responsible for hearing, speech and cognitive impairments in children. *H. influenzae* infections account for about 30% of the cases of acute otitis media and about 60% of chronic otitis media. In the United States alone, treatment of otitis media costs between 1 and 2 billion dollars per year for antibiotics and surgical procedures, such as tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. It is estimated that an additional $30 billion is spent per annum on adjunct therapies, such as speech therapy and special education classes. Furthermore, many of the causative organisms of otitis media are becoming resistant to antibiotic treatment. An effective prophylactic vaccine against otitis media is thus desirable.

(b) The Present Invention

Having regard to the above Background, it would be desirable to provide efficacious combination vaccines comprising *H. influenzae* components containing selected relative amounts of selected antigens. The present invention provides an immunogenic composition for conferring protection in a host against disease caused by infection with *H. influenzae*, including otitis media.

The immunogenic composition comprises at least two different antigens of *H. influenzae*, one of which is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae* at least one of which is an adhesin as claimed in claim 1, and all claims dependant thereon.

Claim 2 recites that the adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *H. influenzae*. Claim 3 recites that the HMW protein is a HMW 1 or HMW 2 protein of a non-typeable strain of *H. influenzae*. Claim 4 recites that the antigen which is not an adhesin is a non-proteolytic heat shock protein of a non-typeable strain of *H. influenzae*. Claim 5 recites that the heat shock protein is an analog of *Haemophilus influenzae* Hin47 protein having a protease activity which is less than about 10% of that of the natural Hin47 protein.

Claim 27 further recites that the Hin47 protein having a decreased protease activity which is less than about 10% of that of the natural Hin47 protein in which the Histidine at amino acid position 91 is replaced by Alanine. The applicants data supports such results.

(c) Rejection of claims 1-5 and 25 under 35 U.S.C. 112 1st paragraph.

The HMW proteins are described in Barenkamp et al cited by the Examiner in the prior art rejection while the Hin47 analogs are described in the Loosmore et al reference cited by the Examiner in the prior art rejection. In the Barenkamp et al reference, there is described both the isolation of the HMW protein from natural-source materials and recombinant production. It is submitted, that the enablement is not limited to recombinantly-produced HMW protein.

In the Loosmore et al reference, there is described the manner of producing the non-proteolytic analog of Hin47 protein. In this respect, at least one amino acid contributing to protease activity is deleted or replaced by a different amino acid. The Loosmore et al reference describes how to identify such amino acid by comparison to known proteases. The reference specifically describes that the deleted or replaced amino acid may be selected from amino acids 195 to 201 and specifically describes replacement of Serine-197 with alanine, other specific amino acid mutations described are Histidine-91 replaced with alanine, and lysine or arginine-121 replaced with alanine. The immunogenic properties of these various mutants are described in Loosmore et al. Based on this information, there is no reason to suppose that any other non-proteolytic analog would not also function in the same manner as the specific H91A Hin47 analog utilized in the experiments described in the application (page 18 lines 4-12). It is submitted that enablement is not limited to the specific H91A Hin47 analog, but rather extends at least to any non-proteolytic analog of the Hin47 protein. The Examiner indicates that the objection of lack of enablement is based, to some extent, upon lack of guidance as to how to determine compositions other than that specifically identified by the Examiner. It is submitted that such is not the case.

Specifically, the specification tells a person skilled in the art that two different antigens of *Haemophilus influenzae* are employed and that one of them has to be an adhesin and the other does not. Testing to determine if an antigen is an adhesin or not an adhesin is within the skill of the art. In this regard, the Examiner's attention is directed to the experimentation described in Barenkamp.

In addition, the person skilled in the art is advised that one such adhesin protein is the HMW protein, where that is described and how to produce it both from natural-source materials and recombinantly (see page 2, line 23 to page 3, line 23). In addition, the person skilled in the art is advised that one such nonadhesin protein is a non-proteolytic analog of Hin47 protein or other non-proteolytic heat shock protein and how to produce such an analog (see page 3, line 14 to page 3 line 31).

Furthermore applicants have given guidance to one skilled in the art to test if a composition is an immunogenic composition (see example 4 pages 18 to 20 of the present application).

Having regard to the foregoing discussion, it is submitted that claims 1 to 5 and 25 are fully enabled by the disclosure.

(d) Rejection of claims 6-24 under 35 USC 103(a).

Claims 1 to 5, 25 and 27 have been finally rejected under 35 USC 103(a) as being unpatentable over Barenkamp (WO 97/36914) in view of Loosmore et al (US Patent 5,506,139).

Claim 1 defines an immunogenic composition for conferring protection in a host against disease caused by *Haemophllus influenzae* comprising at least two different antigens of *Haemophilus influenzae*.

- one of which antigens is an adhesin
- the other of which antigens is not an adhesin.

The Examiner has identified Barenkamp et al as describing a *Haemophilus influenzae* protein which is an adhesin and Loosmore as describing a *Haemophllus influenzae* protein which is not an adhesin. The applicants position is that neither reference provides the motivation to combine the two immunogens in a single composition, as required by claim 1.

Barenkamp teaches high molecular weight proteins of non-typeable *H. influenzae* identified as HMW1, HMW2, HMW3 and HMW4, which are characterized by molecular weight and sequence information. Loosmore et al teach an analog of *H. influenzae* Hin47 protein with reduced protease activity. It is submitted that these references lack any motivation to combine two different antigens of *H. influenzae,* namely a non-proteolytic Hin47 protein of Loosmore et

al with the HMW proteins of Barenkamp et al in an immunogenic composition. It is the applicants position that neither reference provides the motivation to combine the two immunogens in a single composition as required by claim 1, and by dependency all claims on appeal.

While suggesting various combinations, there is no suggestion here to combine different proteins derived from the same pathogen, as in applicants claim 1. Again, the references are silent as to any specific combination contemplated.

The cited prior art lacks the motivation to do so. There are vague, non-specified indications in both references to combine other components with the specific immunogen, but there is no specific indication as to what that other component may comprise, other than an adjuvant or materials from the pathogens and/or materials from various strains of the same pathogen.

As the Examiner has pointed out, on page 49, lines 15 to 19 of Barenkamp, it is stated:

> "…. the data suggests the HMW adhesin proteins are potentially important protective antigens which may comprise one component of a multi-component NTHI vaccine."

This passage appears to suggest that only *Haemophilus* proteins which are the HMW adhesin proteins are appropriate components. The non-proteolytic analog of Hin47 is not an adhesin (although initially thought to be adhesin, see col. 2, line 17 of Loosmore et al). (It is pointed out that the Examiner is incorrect in the statement that the adhesin protein "should" comprises one component of the NTHI vaccine. As can be seen from the above quotation, Barenkamp uses the word "may").

Even if the Examiner finds motivation in this passage of Barenkamp to combine the HMW protein with another *Haemophilus* antigen, whether an adhesin or not, such motivation still provides no motivation to select the non-proteolytic Hin47 analog as the other *Haemophilus* antigen.

There have been a significant number of *Haemophilus* proteins identified as vaccine candidates besides the HMW and Hin47 analog proteins. These proteins include the

various outer membrane proteins A to H, lactoferrin and transferrin receptor protein and the P1, P2, P6 and D15 proteins. It is submitted that there is no motivation provided by the cited prior art why a person skilled in the art would specifically select from all the optional possibilities, the non-proteolytic Hin47 analog to specifically combine with the HMW protein.

The Examiner states in the Office Action, quoting In re Kerkhoven, that:

> "The idea of combining them flows logically from their having been individually taught in the prior art."

The "idea of combining them" does not explain why the two materials should be combined when there is selection available. If the two antigens were the only two known antigens of *Haemophilus influenzae,* then there may be some validity to the position taken by the Examiner, but this is clearly not the case here.

In any event, caution is required when considering combining different antigens into immunogenic compositions because of the danger of impairment of the immunogenicity of the individual components one by the other. As may be seen from Applicants data, in Figure 3, immunogenic compositions are provide in which there is no impairment of individual antigenic components.

Furthermore, these results are unexpected in the field of combination vaccines. There is little expectation of success that simply mixing existing vaccine antigens will not result in incompatibilities amongst the various antigens, resulting in loss of stability or reduced potency or indeed a synergistic effect increasing potency. Immune interference cannot be predicted. Others skilled in the art of combination vaccines have found that the preparation of combination vaccines is far from straight forward. For example Cauldfield et al (2001) report on the need for a balanced formulations of vaccine components in the preparation of DTP combination vaccines to circumvent interference with the components. Van den Bosch et al (2003) have also reported that the addition of a potential antigen (Pal A) from *Actinobacillus pleuropneumoniae* can completely eliminate the positive efficacy of known antigens (Apxl and II) when combined (see abstract).

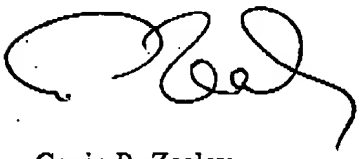
For all these reasons, it is submitted that claims 1 to 5, 25 and 27 are patentable over the applied art and the rejection thereof under 35 USC 103(a) as being unpatentable over

Barenkamp in view of Loosmore et al. cannot be sustained.

Summary

Having regard to the above detailed discussion, it is submitted that the Examiner is in error in rejecting claim 1 to 5, 25 and 27 as being unpatentable under 35 USC 112 1st paragraph and the rejection under 35 USC 103(a) as being unpatentable over the combination of Barenkamp in view of Loosmore et al, should be REVERSED.

Respectfully submitted,



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(9) **APPENDIX I**

CLAIMS APPEALED (09/857,843)

1. An immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae,* comprising:
at least two different antigens of *Haemophilus influenzae,* at least one of which antigens is an adhesin.

2. The immunogenic composition of claim 1 wherein said antigen which is an adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae.*

3. The immunogenic composition of claim 2 wherein said HMW protein is a HMW 1 or HMW2 protein of the non-typeable strain of *Haemophilus influenzae.*

4. The immunogenic composition of claim 1 wherein the antigen of *Haemophilus influenzae* which is not an adhesin is a non-proteolytic heat shock protein of a strain of *Haemophilus influenzae.*

5. The immunogenic composition of claim 4 wherein the non-proteolytic heat shock protein of a strain of *Haemophilus influenzae* is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein.

25. A method of immunizing a host against disease caused by infection with *Haemophilus influenzae,* including otitis media, which comprises administering to the host an immunoeffective amount of a composition as claimed in claim 1.

27. The composition of claim 1 wherein said antigen which is an adhesin is a recombinantly-produced high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus*

influenzae and said antigen which his not an adhesin is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein, in which Histidine-91 is replaced by alanine.

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Immunogenicity of a hexavalent combination vaccine in rhesus monkeys

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Abstract

Preclinical immunogenicity studies were conducted in rhesus monkeys to determine whether there is immune interference in the response to one or more components of a hexavalent vaccine (Hexavac™) that contains antigens from *Haemophilus influenzae* (Hib), hepatitis B (HB), diphtheria (D), tetanus (T), acellular pertussis (aP) and inactivated polio virus (IPV). Antibody responses were measured following co-administration of the components at three separate anatomical sites or administration as a hexavalent combination in a single site. After three injections of the hexavalent vaccine, the peak antibody responses to each component of the vaccine were > 100-fold above pre-immune titers and persisted at levels > 10-fold above pre-immune titers at ≈ 1 year. Immune interference was observed in the peak response to HB, D and pertussis toxin, but was not seen at later time points. The results indicate that the rhesus monkey model may be useful for pre-clinical evaluation of combination vaccines.

Keywords: Combination vaccine; Immune interference; Antigen competition; Non-human primate

1. Introduction

The rationale for the development of combination vaccines has been discussed in recent publications [1–3]. The main benefits are to enhance compliance and vaccine coverage and to reduce overall healthcare costs. An added benefit is to 'make room' in the pediatric vaccination schedule for new vaccines projected for the new millennium [4]. However, experience has shown that the preparation of combination vaccines is far from straightforward. During the development of the DTP combination vaccine, the need for 'balanced' formulations of vaccine components was recognized [5] and careful dose-ranging of the three serotypes of the oral poliovirus vaccine (OPV) was required in order to circumvent interference of the type 2 strain on the immune response to types 1 and 3 [6]. The main impediments to the development of combination vaccines are stability and immunogenicity. Simply mixing existing vaccines can result in incompatibilities among the various antigens, adjuvants, preservatives, stabilizers and excipients, resulting in a loss of stability or reduced potency [7]. A further confounding factor is that of immune interference (also known as antigen competition) which may not always be predicted using animal models. Antigenic competition was first described by Michaelis in 1904 [8], but is still poorly understood.

The objective of the present preclinical immunogenicity studies of Hexavac™ was to determine the antibody response to vaccine component antigens at various times after immunization and to compare the response to the hexavalent vaccine with that induced by administration of Hib, HB and DTaP-IPV at separate anatomic sites. The results indicate that there was a significant difference between experimental and control arms in th peak responses to HB, D and PT. These differences fade with time and there was no significant

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difference in the response to any vaccine component tested at 48–51 weeks post-dose 1. Overall, strong antibody responses were induced to each component of Hexavac™ in rhesus monkeys.

2. Materials and methods

2.1. Experimental animals

The rhesus monkeys (*Macaca mulata*) used in this study were born at the California Regional Primate Center at the University of California at Davis and all immunizations and blood collection procedures were performed at that site. Some monkeys were housed outdoors in social groups, whereas others were maintained indoors, in pairs. Those maintained indoors had a 12:12 h light:dark cycle within a temperature range of ≈17–29°C. Animals were all fed Purina Monkey Chow, 15% protein with fresh produce supplements two to three times per week. Monkeys were identified by tattoos containing unique numbers. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Vaccine composition

The hexavalent vaccine consisted of Hib capsular polysaccharide (polyribosyl ribitol phosphate) conjugated to tetanus toxoid (PRP-T), hepatitis B (HB), surface antigen (HBsAg), diphtheria toxoid (D), tetanus toxoid (T), pertussis filamentous hemagglutinin (FHA), pertussis toxoid (PT) and three serotypes of inactivated poliovirus (IPV) formulated with aluminum adjuvants. Each 0.5 ml dose contained 12 μg PRP-T (expressed in polyoside), 5 μg HBsAg, 30 Lf D, 10 Lf T, 25 μg FHA, 25 μg PT, IPV type 1 (40 D-Ag U), type 2 (8 D-Ag U) and type 3 (32 D-Ag U).

2.3. Vaccination/schedule

One group of rhesus monkeys was immunized with half of the human pediatric dose of the hexavalent combination vaccine (Hexavac™) into a single i.m. site, while a second cohort (control group) of monkeys was injected with half of the human dose of PRP-T (ActHIB®), HBsAg (RECOMBIVAX HB®) and DTacP-IPV at three separate i.m. sites at 0, 4 and 8 weeks, according to the protocol shown in Table 1. The monkeys were weighed at each time point and examined for injection site reactions after each dose of vaccine. In addition, blood samples collected at each time point were monitored for changes in white cell, red cell and platelet levels.

2.4. Serological assays

Sera were collected at week 0, 4, 8, 10 and 48–51 and tested individually for antibody titers against PRP, HBsAg, D, T, FHA and PT. Due to a shortage of sera, serology was not performed to detect antibodies against poliovirus. Anti-PRP (component of Hib) antibody titers were measured using a Farr-type radioimmunoassay (RIA), as previously described [9,10]; responses to HB were determined using a modified Ausab® assay (Abbott Laboratories, N. Chicago, IL), as described elsewhere [11]. Antibodies against FHA [12] and antibody titers against T were measured by ELISA [13]. Diphtheria toxoid antibody titers were assayed by using a neutralization test in comparison to a WHO antitoxin standard [14]. PT antibody titers were also determined by a toxin neutralization test on CHO cell culture [15]. The results are expressed as geometric means. The assays used were originally validated for analysis of human samples and adapted for testing monkey serum without further analytical validation.

2.5. Statistical analysis

At each time point, the estimated GMT ratio for the experimental vaccine group relative to the control group (Hexavac™/Hib + HB + DTaP-IPV) and corresponding two-sample 99% confidence interval for the true GMT ratio are calculated for the response to each antigen, assuming unknown but equal variances between the two groups. If a particular interval excludes

Table 1
Protocol for preclinical immunogenicity testing of Hexavac™ in rhesus monkeys

Group	*n*	Age at dose 1 (months)	Vaccine	Injection schedule (weeks)	Bleeding schedule (weeks)
1	8[c]	6–12	Hexavac™[a]	0, 4, 8	0, 4, 8, 10, ≈ 50
2	8	4.8–10.5	ActHIB®[a] + RECOMBIVAX HB®[a] + DTaP-IPV[b]	0, 4, 8	0, 4, 8, 10, ≈ 50

[a] Injection volume of 0.25 ml in one intramuscular site.
[b] Injection in three separate intramuscular sites (0.25 ml each).
[c] Serum samples from six of eight monkeys were available at the week ≈ 50 time point.

the value 1, the corresponding comparison between Hexavac™ and Hib + HB + DTaP-IPV is statistically significant; otherwise, it is not. The reason for using a 99% confidence level instead of the usual 95% l vel is to control the overall false–positive rate (per antigen), which is defined as the probability that at least one of the confidence intervals will exclude the value 1 by chance alone [16].

3. Results

3.1. Serum antibody response to vaccination

Antibody titers to each component of the vaccine (except IPV) were measured at week 0, 4, 8, 10 and 48–50 using sera from individual animals (Fig. 1). At week 4 (post-dose 1), there were no significant differences between groups in antibody titers to any of the antigens tested. Similarly, at week 8 (4 weeks post-dose 2), there were no significant differences among groups with the exception of the response to HB, which was significantly higher in the control group compared with the monkeys injected with the hexavalent combination. As shown in Table 2, at the 10-week time point (2 weeks post-dose 3), there was a significantly higher response to three of the vaccine components (HB, D and pertussis toxin) in monkeys immunized with separate injections of Hib + HB + DTaP-IPV compared with the response of monkeys immunized with the hexavalent combination vaccine. Importantly, at the final time point (week 48–51), which is 38–41 weeks post-dose 3, there was no significant difference in the response to any component of the vaccines.

3.2. Response rate to vaccination

The percentage of responders to components of the vaccine was determined at each bleed time point. In the absence of established 'seroprotective titers' for rhesus monkeys, the accepted human equivalents were used as shown in the legend to Table 3. For pertussis, there is no proven correlate of protection established for humans, therefore, the percentage of seroconverters was used instead. As shown in Table 3, there was no difference in the response rate to vaccination with Hexavac™ compared with separate site administration of Hib + HB + DTaP-IPV except for the response to HB at the 8-week time point. At that time, only 37% (3/8) monkeys responded to Hexavac™ whereas 100% (8/8) responded to the control. These results are consistent with the analysis of the serological titers that showed a significant difference in anti-HBs titers at this time.

3.3. Adverse event monitoring

Animals were monitored for changes in weight or blood cell counts as well as for injection site reactions. No adverse reactions were noted at the site of injection at any time point and there was no adverse effect of vaccination on the weight or blood cell counts of any animals (data not shown).

4. Discussion/conclusion

The results from the present pre-clinical evaluation of Hexavac™ indicate that there was a vigorous response to each component of the vaccine. Even so, there was evidence for interference in the peak response to HB, D and pertussis toxoid when the responses to Hexavac™ were compared with the control group. Four types of immune interference (antigen competition) have been described: (a) sequential; (b) intramolecular; (c) intravirionic; and (d) intermolecular competition.

- Sequential competition occurs when a second antigen (or vaccine) is given shortly after a first antigen (or vaccine) [17]. This form of interference is especially relevant to vaccine dosing schedules.
- Intramolecular competition results from competition among peptides derived from the same protein for binding to MHC Class I or Class II molecules [18].
- Intravirionic competition results when one protein antigen within a virus interferes with the response to a second protein antigen within the same virus [19]. This form of antigen competition can be circumvented by dissociation of the virus into its component parts prior to immunization.
- Intermolecular competition results when one antigen in a mixture interferes with the immune response to a second antigen in a mixture [17,20]. This form of interference is most relevant to the present investigation; however, the mechanism by which this happens is unknown. One possibility is that one or more components within the combination vaccine become unstable, perhaps due to excipients carried over into the vaccine with a separate component. However, extensive stability studies have been performed on Hexavac™ and the components that had reduced immunogenicity in the combination vaccine (HB, D and PT) were shown to be stable for several years (data not shown). Thus, the decreased response to certain of the vaccine components of Hexavac™ does not appear to be related to a loss of stability of these components, suggesting that the explanation is immune interference due to intermolecular antigen competition.

Although the rhesus monkey model suggests that the response to Hexavac™ is marked by transient interference in response to the HB, D and PT components,

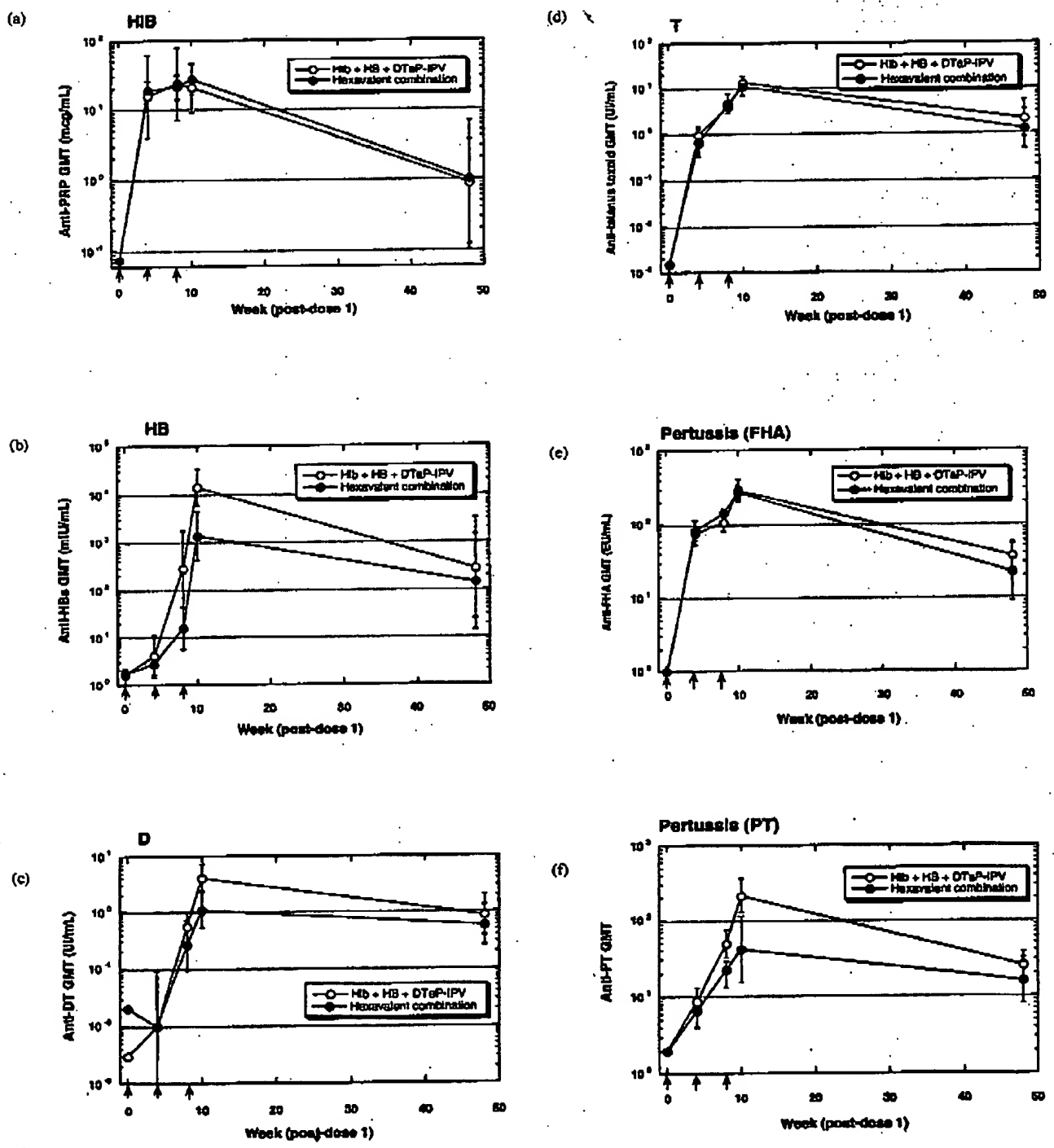

Fig. 1. Antibody response to antigens from Hib, HB, D, T, FHA and PT in rhesus monkeys immunized with Hib + HB + DTaP-IPV at separate sites or with the hexavalent combination vaccine (Hexavac™) at a single i.m. site. Monkeys were immunized at week 0, 4 and 8 and antibody titers were determined on serum collected at week 0, 4, 8 and ≈ 50. Results are expressed as the geometric mean with 95% confidence intervals.

these apparent differences in potency are unlikely to translate into clinically meaningful differences since: (a) the response to each component of the vaccine is > 100-fold higher than the pre-immune titers; (b) th response rate post-dose 3 (percent seroconverters) is equivalent in the two groups; and ( ) the difference in titers elicited by Hexavac™ and the control arm became indistinguishable over time. By study week 48–51,

Table 2
Estimated ratios (Hexavac™/Hib+HB+DTaP-IPV) and 99% confidence intervals for the antibody response to Hexavac™ versus control[a]

Antigen	Week (post-dose 1)			
	4	8	10	48–51
HB	0.69 (0.15, 3.21)	**0.06 (0, 0.8)**	**0.1 (0.02, 0.063)**	0.5 (0.01, 37.61)
Hib	1.21 (0.2, 7.16)	0.9 (0.23, 3.56)	0.76 (0.23, 2.56)	2.24 (0.14, 36.74)
D	1.49 (0.09, 25.11)	0.47 (0.1, 2.1)	**0.27 (0.08, 0.86)**	0.66 (0.14, 3.04)
T	0.69 (0.25, 1.95)	1.17 (0.55, 2.49)	0.83 (0.39, 1.79)	0.61 (0.11, 3.4)
FHA	1.08 (0.58, 2.03)	1.31 (0.88, 1.95)	0.94 (0.57, 1.57)	0.6 (0.2, 1.82)
PT	0.77 (0.38, 1.55)	0.46 (0.19, 1.09)	**0.19 (0.05, 0.82)**	0.65 (0.26, 1.64)

[a] Any confidence interval excluding the value '1' implies a statistically significant difference. If the upper confidence limit is <1, then the GMT of Hexavac™ is significantly less than the GMT of Hib+HB+DTaP-IPV. Statistically significant results are in bold type.

Table 3
Response rate to Hexavac™ versus the control group[a]

Antigen	Percent responders (Hexavac™, control) at week:				
	Pre	4	8	10	48–51
HB	0, 0	0, 12	37, 100	100, 100	83, 87
Hib	12, 12	100, 100	100, 100	100, 100	83, 75
D	33, 0	17, 0	100, 100	100, 100	100, 100
T	0, 0	0, 0	100, 100	100, 100	100, 100
FHA	0, 0	0, 0	100, 100	100, 100	100, 100
PT	0, 0	62, 87	100, 100	100, 100	100, 100

[a] Criteria for response: HB (>10 mIU/ml); Hib (>0.15 mcg/ml); D (>0.01 IU/ml); T (>0.01 IU/ml); FHA (>4 UE/ml); PT (reciprocal titer >4).

there was no significant difference in the antibody response to any component of the vaccine between monkeys injected with Hexavac™ versus separate site injection of Hib + HB + DTaP-IPV. The kinetics of the response also deserves comment. With the exception of the response to Hib, the titers to each vaccine component increased following each dose of vaccine. The reason for the immediate and vigorous response to Hib is not known, however, the rapid response suggests that the monkeys may have been primed by prior exposure to *Haemophilus influenzae* or to a cross-reacting organism. Subsequent injections of Hexavac™ did not significantly increase the high titers observed after the first injection, which were > 10 μg/ml. In previous studies [10], 18–22-month-old rhesus monkeys were found to respond earlier and with higher titers to a Hib vaccine (PedvaxHIB®) than 2–3-month-old monkeys. This suggests that the use of younger monkeys may enable better discrimination among Hib-containing vaccines.

The response rate of rhesus monkeys that received three doses of the vaccine was 100% (at week 10). This compares favorably with the response rates seen in a clinical trial of Hexavac™ in which the response rate was 87–100% for each of th antigens [3]. However, the antibody tit rs achieved in rhesus monkeys were ≈10-fold higher that titers attained in the human post-dose 3. The magnitude of the response of th rhesus monkeys to three doses of Hexavac™ was similar to that of human infants given four injections of Hexavac™ [3]. It should be noted, however, that the small sample size ($n = 8$ monkeys per group) is a limitation in the interpretation of antibody titers and response rates. With these caveats, the rhesus monkey animal model described herein may be useful in the evaluation and development of future combination vaccines.

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References

[1] Ellis RW. Development of combination vaccines. Vaccine 1999:17:1635.
[2] Andre FE. Development and clinical application of new polyvalent combined paediatric vaccines. Vaccine 1999;17:1620.
[3] Pines E, Barrand M, Fabre P, Salomon H, Blondeau C, Wood SC, et al. New acellular pertussis-containing paediatric combined vaccines. Vaccine 1999;17:1650.

[4] Eskola J. Epidemiological views into possible components of paediatric combined vaccines in 2015. Biologicals 1994;22:323.
[5] Barr M, Llewellyn-Jones M. Some factors influencing the response to immunization with single and combined prophylactics. Br J Exptl Pathol 1955;36:147.
[6] Plotkin S, Koprowski H, Stokes J. Clinical trials in infants of orally administrated attenuated poliomyelitis viruses. Pediatrics 1968;23:1041.
[7] Corbel MJ. Control testing of combined vaccines: a consideration of potential problems and approaches. Biologicals 1994;22:353.
[8] Michaelis L. Weitere untersuchungen uber eiweißprazipitine. Dtsch Med Wschr 1904;30:1240.
[9] Kuo J, Monji N, Schwalbe R, McCoy D. A radioactive antigen binding assay for the measurement for the measurement of antibody to haemophilus influenzae type b capsular polysaccharide. J Immunol Methods 1981;43:35.
[10] Vella P, Staub J, Armstrong J, Dolan K, Rusk C, Szymanski S, et al. Immunogenicity of a new *Haemophilus influenzae* type b conjugate vaccine (Meningococcal protein conjugate) (Pedvax-HIB®). Pediatrics 1990;85:S668.
[11] Wang S, Liu X, Fisher K, Smith JG, Chen F, Tobery TW, et al. Enhanced type I immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminum-phosphate. Vaccine 2000;18:1227.
[12] Manclark C, Meade B, Burstyn D. Serological response to Bordatella pertussis. In: Rose N, Friedman H, Fahey J, editors. Manual of Clinical Laboratory Immunology, 3rd ed. Washington, DC: American Society for Microbiology, 1986:388.
[13] Bizzini B. Tetanus. In: Germanier R, editor. Bacterial Vaccines. Orlando, FL: Academic Press, 1984:37.
[14] Miyamura K, Nishio S, Ito S, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using Vero cells: I. Studies on factors affecting the toxin in microplate culture Chinese Hamster Ovary cells. J Biol Stand 1974;2:189.
[15] Gillenius P, Jaatmaa E, Askelof P, Granstrom M, Tiru M. The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese Hamster Ovary cells. J Biol Stand 1985;13:61.
[16] Fisher LD, van Belle G. Biostatistics: A Methodology for the Health Sciences. New York: Wiley, 1983.
[17] Taussig MJ. Antigenic competition. Curr Topic Microbiol Immunol 1973;60:125.
[18] Babbit BP, Matwueda G, Haber E, Unanue ER, Allen PM. Antigenic competition at the level of peptide-Ia binding. Proc Natl Acad Sci USA 1986;83:4509.
[19] Johansson BE, Moran TM, Kilbourne ED. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. Proc Natl Acad Sci USA 1987;84:6869.
[20] Pross HF, Eidinger D. Antigen competition: a review of nonspecific antigen-induced suppression. Adv Immunol 1974;18:133.

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Interference of outer membrane protein PalA with protective immunity against *Actinobacillus pleuropneumoniae* infections in vaccinated pigs

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Abstract

The role of antibodies to the outer membrane protein PalA of *Actinobacillus pleuropneumoniae* in protective immunity was studied in pigs vaccinated with purified PalA alone and PalA in combination with toxoids of the RTX toxins ApxI and ApxII using an established challenge model with the virulent serotype 1 of *A. pleuropneumoniae*. Pigs that developed antibody titers against PalA after immunization were more significantly affected by challenge with *A. pleuropneumoniae* serotype 1. Following challenge, pigs that were immunized with PalA showed more severe respiratory symptoms, had a higher mortality rate and died faster. They also displayed much more severe lung lesions after necropsy than animals not immunized with PalA. Pigs that were immunized with toxoids of the two cytotoxins ApxI and ApxII were protected against challenge with *A. pleuropneumoniae*. In contrast, the protective efficacy of the ApxI and ApxII vaccine was completely lost when it was supplemented with PalA. Hence, antibodies induced against the outer membrane protein PalA of *A. pleuropneumoniae* aggravated the consequences of infection and counteracted the protective effect of anti-ApxI and anti-ApxII antibodies. Due to the high similarity between protein analogues of PalA from various bacteria of the *Pasteurellaceae* family such as P6 of *Haemophilus influenzae* or 16 kDa Omp of *Pasteurella multocida*, this deleterious effect of PalA in vaccination should be taken into consideration in the development of vaccines against infections with other *Pasteurellaceae*.

Keywords: Outer membrane protein; Experimental infection; Antigenicity; Immunity; Vaccine; Porcine pleuropneumonia; *Pasteurellaceae*

1. Introduction

Actinobacillus pleuropneumoniae, a Gram-negative bacterium of the family *Pasteurellaceae*, is the etiological agent of porcine pleuropneumonia, a severe contagious disease of swine with worldwide prevalence [1]. To date, fifteen serotypes have been described which variously express three different cytotoxins belonging to the RTX toxin family: ApxI, ApxII, ApxIII [2]. These toxins mainly determine the virulence of the different serotypes [3]. A fourth RTX-toxin, ApxIV, which is expressed by all serotypes of *A. pleuropneumoniae*, was recently detected and shown to be produced during infection but not during culture in growth medium [4,5]. Serotypes 1, 5a/b, 9 and 11, which express ApxI, ApxII and also ApxIV, are particularly virulent, while the other serotypes which are devoid of ApxI are generally less virulent [3]. Vaccination is an effective strategy for the prevention of porcine pleuropneumonia outbreaks. Recently, a new generation subunit vaccine, composed of the three major RTX exotoxins (ApxI, ApxII and ApxIII) and a 42 kDa outer membrane protein of *A. pleuropneumoniae*, has been developed and shown to give high protection against all 12 major serotypes (serotypes 1–12) under experimental conditions [6,7] as well as in field trials [8–10]. Vaccination of pigs with the RTX toxins alone protects against mortality but generally fails to reduce the typical *A. pleuropneumoniae* lung lesions, while the combination of RTX toxins with the 42 kDa outer membrane protein (42 kDa OMP) induced complete protection with regard to mortality as well as lung lesions [6]. The 42 kDa OMP is therefore a valuable component of the vaccine. Among the OMPs of *A. pleuropneumoniae*, PalA is the most immuno-predominant antigen [11,12]. PalA is a 14 kDa protein, encoded by *palA* as a precursor peptide which is processed by signal sequence peptidase II and sorted by a peptide located signal

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for the final localization of the mature protein in the outer membrane. PalA is conserved in all *A. pleuropneumoniae* serotypes and induces a strong IgG response in pigs naturally or experimentally infected with *A. pleuropneumoniae* [11]. PalA shows high amino-acid (aa) sequence homology to the family of peptidoglycan associated proteins (PAL) of Gram-negative bacteria showing most similarity to the P6 protein of *Haemophilus influenzae* [11,13,14] and to the 16 kDa Omp of *Pasteurella multocida* [15]. There is evidence that PAL proteins of *Pasteurellaceae*, in particular P6 of *H. influenzae*, can act as protective antigens. P6 was therefore suggested to be included in vaccines against *H. influenzae* induced meningitis and avian cholera [15,16]. Antiserum directed against recombinant P6 protein was shown to be bactericidal against clinical *H. influenzae* isolates, including highly pathogenic non-typable strains [17]. Antibodies to P6 give passive protection to infant rats against *H. influenzae* type b-induced meningitis. In the view of the importance of PAL proteins as potential vaccine antigens, we have designed the present study to analyze the role of PalA alone, and in combination with toxoids of the RTX toxins ApxI and ApxII in induction of protective immunity against challenge of pigs with a virulent *A. pleuropneumoniae* serotype 1 strain, 4074^T.

2. Materials and methods

2.1. Bacterial strains, growth conditions and vectors

A. pleuropneumoniae 4074^T (serotype 1 reference strain) used for PCR amplification of the *palA* gene, and *A. pleuropneumoniae* serotype 1 strain 1-L-452 used for challenge, were grown on solid Columbia broth agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10 mM β-NAD (Sigma Chemicals, St. Louis, MO, USA) or in liquid medium consisting of Columbia broth supplemented with 10 mM β-NAD. In order to avoid appearance of contaminating bacteria during re-isolation of *A. pleuropneumoniae* from the challenged pigs, lungs and tonsils were first superficially decontaminated with a hot spatula before sampling with sterile tools from the inside of the tissues.

Escherichia coli K-12 strain XL1-blue MRF′ (Stratagene, La Jolla, CA, USA) and *E. coli* B strain BL21 (DE3) (Novagen, Madison, WI, USA) were grown in Luria-Bertani (LB) broth [18] at 37 °C in orbital shaker incubator. Ampicillin 100 μg/ml was added when needed for selection or stabilization of plasmids. Cloning vector pETHIS-1 [4] was used for the production of recombinant poly-histidine tailed peptides.

2.2. DNA extraction, manipulation, cloning and sequence analysis

Genomic DNA from *A. pleuropneumoniae* was extracted by th guanidiumthiocyanate method [19]. Ligation, gene cloning, plasmid extraction, restriction endonuclease digestion and analysis of the DNA fragments by agarose gel electrophoresis were performed using standard protocols [18]. Plasmid extraction was done using the alkaline lysis method with the Miniprep kit (Qiagen AG, Basel, Switzerland). DNA sequencing reactions were performed with approximately 500 ng plasmid DNA per reaction mixture and 5 pmol of primer. Sequences were determined with an ABI Prism model 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled and edited by using the Sequencher 3.0 program (Gene Codes Corp., Ann Arbor, MI, USA) to obtain contiguous sequences.

2.3. Production of antigens for vaccines

PalA protein from *A. pleuropneumoniae* serotype 2 strain S411 for vaccination was produced as described earlier and involved heat extraction in PBS buffer (Na-phosphate 50 mM, pH 7.5, NaCl 0.85%) for 1 h at 60 °C, ammonium sulfate fractionation and separation by SDS-PAGE followed by electroelution [11]. The commercially available subunit vaccine, Porcilis App™ (Intervet International, Boxmeer, the Netherlands), was used as reference preparation for the titration of the ApxI and ApxII antigens. ApxI and ApxII proteins were prepared from supernatants of *A. pleuropneumoniae* serotype 5b bacteria grown at 37 °C for 6 h in Columbia broth supplemented with βNAD 10 μg/ml and $CaCl_2$ 25 mM. Cells were removed by centrifugation and sterile filtration through a low protein binding 0.45 μm filter (Acrocap, #4482, Gelman Laboratory, Ann Arbor, MI, USA). ApxI and ApxII were subsequently concentrated by ultrafiltration on a 500 kDa MW cut-off polyether sulfon filter (Amicon, bioseparations, Millipore, Bedford, MA, USA). Protein concentrations were measured by the method of Bradford [20]. ApxI and ApxII preparations were analyzed by standard SDS-PAGE stained with Coomassie blue [21] where they revealed a predominant band at 105 kDa indicating that ApxI and ApxII represented the major proteins (estimated to 90% of the total proteins) in the preparations [22].

For the vaccination of pigs, the following vaccines were produced: (i) Vaccine I (PalA) consisted of 80 μg PalA protein in 2 ml Diluvac Forte® adjuvant formulation (Intervet International) per vaccine dose with 0.02% (v/v) formaldehyde final concentration. (ii) Vaccine II (PalA + ApxI + ApxII) consisted of 80 μg recombinant PalA, ApxI and ApxII in 2 ml Diluvac Forte® adjuvant formulation per vaccine dose with 0.02% (v/v) formaldehyde final concentration. (iii) Vaccine III (ApxI + ApxII) consisted of ApxI and ApxII in 2 ml Diluvac Forte® adjuvant formulation per vaccine dose with 0.02% (v/v) formaldehyde final concentration; the concentrations of ApxI and ApxII in vaccines II and III were titrated by specific antigenic mass ELISA and adjusted to the same concentrations of antigens in the commercially available vaccine Porcilis App™ corresponding to approximately 50 μg of ApxI and 25 μg f ApxII per dose

(2 ml) of vaccine. (iv) Vaccine IV (Porcilis App™) was a positive protection control, using the commercially available subunit vaccine Porcilis App™ (Intervet International).

2.4. Vaccinations and challenge

Vaccination was done intramuscularly. For each vaccine, three 6 weeks old SPF landrace pigs were immunized on day 0 (zero) and subsequently received a booster vaccination on day 28. Three control animals (group V) received injections of adjutants on the same days. Two weeks after booster vaccination, on day 42, control sera were taken and then the pigs were challenged with *A. pleuropneumoniae* serotype 1 strain 1-L-452. The bacteria for challenge were grown for 6 h and washed twice by centrifugation at 10,000 × *g* for 15 min and re-suspension in the original volume of PBS buffer. Pigs of all groups were exposed at the same time for 15 min to the bacteria in an aerosol using a De Vilbiss nebulizer [23]. After challenge, fever and respiratory symptoms were recorded. Two weeks later, on day 56, the pigs were slaughtered, unless they died of the infection or had to be euthanized prior to this date. Blood was taken on the day of immunization, on day 42 (prior to challenge) and from the surviving animals at day 56. Dead and euthanized animals were subjected to post-mortem examination for typical lung lesions and recovery of challenge bacteria from lungs and tonsils.

2.5. Production of recombinant PalA'His

In order to specifically monitor the antigenic response to PalA in vaccinated pigs, we have produced an antigenically specific recombinant peptide consisting of the hydrophilic central- and C-terminal part of PalA containing the 106 most C-terminal amino acids (11.54 kDa) fused to 6 N-terminal and 10 C-terminal histidine residues. The corresponding part of the *palA* gene was amplified using the oligonucleotide primers X89009-L (cgcc*catatg*-CAAACTCGTTACACCACT) and X89009-R (cgcg*gatcc*-GTATTCTAATACTGCACG). The primers were designed to contain recognition sites for the restriction enzymes *Nde*I and *Bam*HI (shown in italic letters) by the addition of supplementary nucleotides (shown in lower case). This procedure allowed the PCR amplification product to be cloned into the *Nde*I and *Bam*HI cloning sites of vector pETHIS-1. PCR was carried out with a DNA thermal cycler (GeneAmp 9600; Perkin-Elmer Cetus, Norwalk, CT, USA) in a 50 μl reaction mixture containing 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 170 μM of each dNTP, 0.25 μM forward and reverse primers, 0.5 units *Pwo* polymerase and 5 ng genomic DNA of *A. pleuropneumoniae* strain 4074. The amplification was carried out for 35 cycles (30 s denaturation at 94 °C, 30 s annealing at 50 °C, 1 min elongation at 72 °C). The PCR product was purified using the QIA quick PCR purification kit (Qiagen, Basel, Switzerland), cut with the restriction enzymes *Nde*I and *Bam*HI and ligated to *Nde*I and *Bam*HI digested pETHIS-1. Ligated DNA was transformed into *E. coli* strain XL1-blue MRF and positive clones were selected by colony hybridization using a digoxigenin labelled DNA probe for *palA* [11]. A positive clone, named pJFF-PalA1H, was sequenced to verify the integrity of the cloned segment of *palA* and the fusions with the poly-His codons of pETHIS-1. For biosynthesis of recombinant PalA'His peptide, plasmid pJFFPalA1H was introduced into *E. coli* BL21 (DE3) for expression, which was induced by addition of 1 mM IPTG at mid-exponential phase and incubation for a further 2.5 h. Following induction, the poly-histidine tailed fusion protein PalA'His was purified from cell extracts dissolved with 6 M guanidine hydrochloride using Ni^{2+} chelate affinity chromatography (Qiagen) according to the manufacturer's instructions. The bound PalA'His was eluted by slowly decreasing the pH from 8.0 to 4.5 with 50 mM potassium phosphate buffer, 300 mM NaCl, 6 M guanidine hydrochloride. Following elution at pH 4.5, the fusion protein was dialyzed against 50 mM phosphate buffer, 300 mM NaCl, pH 7.5. The fractions were analyzed by standard SDS-PAGE [18] and protein concentrations were measured by the method of Bradford [20].

2.6. Serological assays

In order to monitor the immune response to PalA in vaccinated pigs, we have developed an immunoblot assay using purified recombinant PalA'His as an antigen. Samples of 100 μg PalA'His were separated on 14% SDS-PAGE of 8 cm × 8 cm and subsequently blotted onto nitrocellulose membranes (BioRad, Hercules, CA, USA, product no. 162-0112). The dried membranes were cut into 20 strips with a width of 4 mm in order to get immunoblot strips each containing 5 μg of PalA'His. The strips were reacted with pig sera diluted 1:500, using the standard immunoblot procedure [18]. Phosphatase labelled goat antibodies, directed against pig IgG (Kirkegaard & Perry, Gaithersburg, MD, USA; product no. 051401), diluted 1:2000, followed by addition of nitroblue tetrazolium and bromochlorindolyl phosphate in alkaline phosphate buffer [18], were used to visualize bound antibodies. Each lot of immunoblot strips was controlled using rabbit anti-PalA antibodies [11] at a dilution of 1:1000 and phosphatase labelled goat antibodies directed against rabbit IgG (Kirkegaard & Perry, product no. 075-1506) diluted 1:2000.

Specific antibody titers in serum against ApxI, ApxII, ApxIII and 42 kDa OMP were determined by indirect ELISA as described [24]. This ELISA is based on the antigens ApxI, ApxII, ApxIII and 42 kDa OMP that were purified from *A. pleuropneumoniae* strains with serotypes not related to the production strains used for vaccine antigen production, in order to avoid possible cross-reaction with contaminating polysaccharides. Concentrations of antigen preparations were determined in antigenic mass ELISA (toxins) or SDS-PAGE (OMP), relative to reference

preparations. The antigenic mass ELISA determines the concentration in arbitrary units relative to the reference preparations. The concentrations of the proteins used for coating of the micro-titer plates for antibody ELISA were in the order of 1 μg/ml. Final adjustments were made depending on antibody titers obtained with positive and negative reference sera. Routinely samples were measured twice and the mean values were reported. The differences of the two measurements were below 10%.

3. Results

3.1. Immune response to PalA in vaccinated pigs

The results of the analysis of anti-PalA antibodies in the blood sera by immunoblots with PalA'His are shown in Fig. 1. All pigs were free of anti-PalA antibodies before vaccination. Pigs vaccinated with vaccines I or II, which contained PalA alone or in combination with ApxI + ApxII, showed a weak reaction 28 days after the first vaccination, and a strong anti-PalA reaction 2 weeks after the booster vaccination. One pig belonging to group II (#176), from which a blood sample was taken before slaughter, showed an anti-PalA reaction on day 56 (Fig. 1). Groups III and IV of pigs that were vaccinated with vaccine containing ApxI + ApxII or with the commercial vaccine respectively, did not show anti-PalA antibodies after first vaccination (day 28) or after booster vaccination (day 42). One pig (#181) of group III showed a very weak anti-PalA reaction 2 weeks after challenge on day 56, which is thought to be due to the challenge with *A. pleuropneumoniae* (Fig. 1).

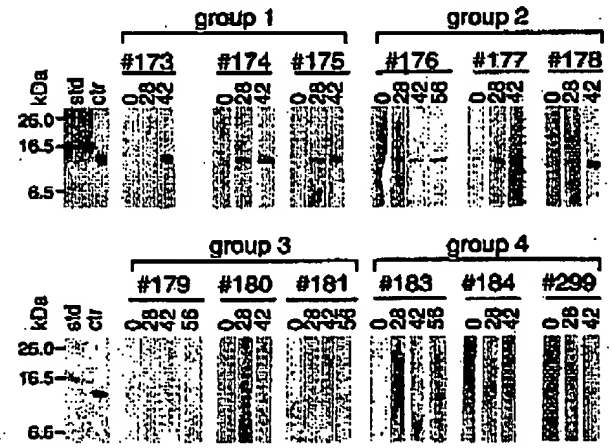

Fig. 1. Serological analysis of anti-PalA antibodies. Immunoblot strips each containing 5 μg PalA'His protein incubated with the different sera before and after vaccination are shown. Figures in the horizontal position indicate the pig numbers, figures in the vertical position indicate days after vaccination. Group I included pigs #173, #174 and #175 vaccinated with vaccine I (PalA); group II included pigs #176, #177 and #178 vaccinated with vaccine II (PalA + ApxI + ApxII); group III included pigs #179, #180 and #181 vaccinated with vaccine III (ApxI + ApxII); group IV included pigs #183, #184 and #299 vaccinated with vaccine IV (Porcilis App™, (Intervet) containing ApxI, ApxII, ApxIII and 42 kDa Omp). Note that pigs #173, #174, #175 and #177 died or were euthanised before the end of the experiment. Std: broad range pre-stained protein markers (New England Biolabs, Beverly, Mass, USA; no. 77085); the position of the molecular masses of 25, 16.5 and 6.5 kDa are indicated.

Table 1
Antibody responses after two vaccinations, measured 2 weeks after booster vaccination and before challenge

Vaccine (content)	Antibody titer against antigen			
	ApxI	ApxII	ApxIII	42 kDa OMP
I (PalA)	<2[a]	<2[a]	<2.0[a]	<2.0[a]
II (PalA + ApxI + ApxII)	3.3	2.6	<2.0[a]	<2.0[a]
III (ApxI + ApxII)	3.5	2.6	<2.0[a]	<2.0[a]
IV (Porcilis App™)	3.2	2.8	2.8	2.4
V (Control)	<2.0[a]	<2.0[a]	<2.0[a]	<2.0[a]

ELISA titers are expressed as the logarithm ($\log_{10}$) of the reciprocal of the highest dilution of serum with an OD above that of the preimmune serum for each pig diluted 1:100 as defined by [24]. Figures represent the mean values of two measurements.

[a] Indicates below detection level.

The antibody responses to vaccination against ApxI, ApxII, ApxIII and 42 kDa OMP were as expected for the various vaccines containing these antigens, as shown in Table 1. The comparison of antibody titers against ApxI and ApxII after vaccination with PalA (group II) or without PalA (group III) added, showed that the serological titers of ApxI and ApxII were not affected by the presence of PalA in the vaccine.

3.2. Protection against infection with A. pleuropneumoniae

The susceptibility of the pigs to *A. pleuropneumoniae* was assessed by challenge of a non-vaccinated group of pigs. In this group, all pigs showed high fever after the challenge as well as abdominal respiration and coughing, which are typical signs for pleuropneumonia (Table 2). One of the three pigs died 6 days post-infection. Upon necropsy, all pigs showed lung lesions affecting, on average, 50–75% of the lungs. These results showed that the challenge with *A. pleuropneumoniae* serotype 1 strain resulted in typical signs of porcine pleuropneumonia under the given experimental conditions.

The group of pigs vaccinated with the commercial vaccine Porcilis App™ (vaccine IV) showed significantly fewer clinical signs after challenge with low or no fever and virtually no respiratory distress. No mortality occurred in this group and no or only minor lung lesions affecting less than 25% of the lung were detected. These results testify to the high protection obtained with the subunit vaccine.

In the group of pigs vaccinated with ApxI and ApxII alone (vaccine III), a high level of protection against infection with *A. pleuropneumoniae* serotype 1 was observed. This was similar to the group vaccinated with the commercial subunit

Table 2
Effect of challenge with *A. pleuropneumoniae* serotype 1 on pigs vaccinated with various vaccines

Vaccine (content)	Pig no.	Fever[a]	Respiratory distress[b]	Mortality (day p.i.)[c]	Lung lesion score[d]	Re-isolation[e]	
						Lung	Tonsil
I (PalA)	173	nd	3	+ (1)	4	+	+
	174	nd	3	+ (1)	4	+	–
	175	2	3	+ (2)	4	+	Cont.
	avg	2	3	3/3 (1.3)	4	3/3	1/2
II (PalA + ApxI + ApxII)	176	2	2	–	2	+	+
	177	0	3	+ (2)	4	Cont.	Cont.
	178	2	2	–	3	+	+
	avg	1.3	2.3	1/3 (2)	3	2/2	2/2
III (ApxI + ApxII)	179	0	0	–	0	–	+
	180	0	0	–	0	–	+
	181	1	1	–	0.5	+	–
	avg	0.3	0.3	0/3	0.2	1/3	2/3
IV (Porcilis App™)	299	1	1	–	1	+	–
	183	0	0	–	0	–	+
	184	1	0	–	1	+	–
	avg	0.7	0.3	0/3	0.6	2/3	1/3
V (Control)	185	2	2	+ (6)	4	+	+
	186	2	2	–	2	–	+
	189	2	2	–	3	+	+
	avg	2	2	1/3 (6)	3	2/3	3/3

[a] Scale used: (0) no fever (<40 °C); (1) fever (>40–41 °C); (2) high fever (>41 °C); (nd) could not be determined due to the rapid death of the animal.
[b] Scale used: (0) no distress, normal respiration; (1) increased respiration rate; (2) abdominal respiration and/or coughing; (3) dyspnea.
[c] (+) Indicates dead pig or pig euthanised because of severe clinical signs (number in brackets gives the day of death post-infection); (–) indicates that the pig did not die.
[d] Scale used: (0) no lesions; (1) 1–25% of the lungs affected; (2) 26–50% affected; (3) 51–75% affected; (4) 76–100% affected (severe lesions).
[e] (+) Indicates animal from which the challenge strain was re-isolated; Cont.: isolation of *A. pleuropneumoniae* not possible since culture was contaminated by other bacteria; (–) indicates that no *A. pleuropneumoniae* could be isolated.

vaccine. This group showed no mortality, only one pig with mild fever and increased respiration rate and virtually no lung lesions after necropsy.

Vaccine II, containing PalA added to ApxI and ApxII, showed no protection. On average, the pigs showed clinical signs similar to the non-vaccinated control group. In this group, the mortality rate was similar to that of the control group. In addition, lung lesions in this group showed the same scores as in the non-vaccinated group.

Vaccination of pigs with purified PalA alone (vaccine I) showed no protection, but rather severe symptoms and rapid death after challenge with *A. pleuropneumoniae* serotype 1. All three pigs died within 1 or 2 days after challenge and subsequent necropsy revealed severe lung lesions. In comparison with the non-vaccinated control group, vaccination of pigs with PalA alone resulted in a significant increase in mortality, three out of three PalA vaccinated animals died, compared to one animal out of three that died from the control group ($\chi^2 = 3.0$; $P = 0.08$) (Table 2). Most significant, was the sudden death after challenge of the PalA vaccinated pigs one to 2 days post-infection, while in the control group one pig died 6 days post-infection (Table 2). Upon necropsy, all pigs vaccinated with PalA showed very severe lung lesions (score 4 with 75–100% of the lungs affected) compared to one animal out of three in the control group (Table 2).

From pigs of all groups, the challenge strain could be re-isolated after necropsy from lungs and from tonsils, showing no differences between the control group and the different vaccinated groups. From a few animals, re-isolation of the challenge strain was hindered due to strong growth of other bacteria on the culture media (Table 2).

4. Discussion

The family of PAL proteins has been characterized as proteins constituting integral parts of the outer membrane of many Gram-negative bacteria. They are highly conserved within given bacterial species. Moreover, they show strong similarities between different bacterial species. They are described as very strongly antigenic proteins in several pathogenic bacteria such as *A. pleuropneumoniae* [11], *H. influenzae* [25,26], *Legionella pneumophila* [27], *P. multocida* [15], *Campylobacter jejuni* [28] and *Brucella abortus* [29]. The prototype of PAL, the peptidoglycan associated lipoprotein PAL of *E. coli* was shown to form complexes,

one type together with the outer membrane proteins TolA, TolQ and TolR, and a second type with the periplasmic protein TolB, in order to maintain the outer membrane integrity by anchoring the outer membrane to the peptidoglycan layer [30]. Mutants deficient in the PAL protein appear to be debilitated for growth under certain conditions. A PAL-deficient mutant of *Haemophilus ducrei* was shown to display a reduced capacity for pustule formation compared to its wild type parent when injected in human volunteers [31]. In addition, the mutant was more susceptible to the antibiotics Erythromycin, Cefotaxime and Ciprofloxacin than the wild type strain and could not be reisolated from pustules in contrast to the wild type strain [31]. The PAL proteins seemed therefore to be valuable targets for immune protection. Several reports of indirect evidence indicate that protein P6 (alternatively named Hi-PAL) of *H. influenzae* is involved in the induction of protective immunity against *H. influenzae* infections. They include studies showing that antibodies to P6 are protective in the infant rat model against invasive *H. influenzae* type b [32]; the demonstration of bactericidal activity for *H. influenzae* of an antibody to P6 immunopurified from human serum [33]; and the fact that rabbit antiserum raised to purified P6 is bactericidal for a broad range of *H. influenzae* strains including many non-typable *H. influenzae* [16]. From these results it was thought that P6 might be a valuable subunit in vaccines against *H. influenzae* infections. Moreover, PAL of other pathogens were considered as appropriate antigens in vaccines. However, no protection against avian cholera was obtained by vaccination with recombinant P6-like protein from *P. multocida* [34].

In our approach to examine the effect of a PAL protein as candidate for a vaccine, we have taken advantage of a well established challenge model of pigs with *A. pleuropneumoniae* to test the efficacy of PalA alone or PalA in combination with known protective antigens as vaccines against porcine pleuropneumonia. In our study, a small number of animals were tested for ethical reasons and, therefore, it does not allow for thorough statistical analysis. However, our data clearly show that pigs, which developed antibody titers against PalA after immunization, showed more significant symptoms, a much higher mortality and died much faster after challenge with *A. pleuropneumoniae* than unvaccinated control pigs. The more severe lung lesions found after necropsy in the PalA vaccinated group further highlighted this observation. Hence the higher mortality and the faster occurring death in the PalA vaccinated group seemed to be an aggravation of the pleuropneumonia and was not due to secondary effects like septic shock. The difference in protective efficacy between the vaccine containing ApxI and ApxII and the vaccine with ApxI, ApxII and PalA is of particular interest. The pigs vaccinated with the two cytotoxins ApxI and ApxII were well protected against challenge with *A. pleuropneumoniae* serotype 1, like the group that was vaccinated with the commercially available subunit vaccine Porcilis App™. In contrast, the protective efficacy of ApxI and ApxII vaccine was completely lost when it was supplemented with PalA, as shown in the group of pigs vaccinated with vaccine II. Hence, PalA antibodies significantly reduce the protective effect of anti-ApxI and anti-ApxII antibodies. The mechanism behind this negative effect of PalA on protective immunity is not known. However, we rule out the possibility that PalA would have had a negative effect on the induction of antibodies against ApxI and ApxII, as the anti ApxI and ApxII titers are the same in the presence or absence of PalA. Since PalA is well conserved in all serotypes of *A. pleuropneumoniae*, the effect is expected to occur with any of the serotypes.

Although the limited number of animals used did not allow us to perform dose-dependence studies, we conclude that PalA should be absent in vaccines against *A. pleuropneumoniae*. Our study does not permit us to extrapolate whether other PAL antigens such as P6 of *H. influenzae* would yield similar effects. However, PalA shows very high similarity to P6 of *H. influenzae* (73% identical and 82% similar aa) and to P6-like protein of *P. multocida* (72% identical and 97% similar aa). Vaccination with other PAL proteins could therefore result in similar negative effects, or as in the case of the P6-like protein of *P. multocida*, give no protection [34]. When using whole cell preparations of bacterial cultures (bacterin vaccines), it must be noted that the concentration of PAL proteins varies depending on the mode of cultivation and preparation of the bacteria, and might therefore vary from one batch to another. Consequently, this could be an explanation of the variations in protective efficacy of certain bacterin vaccines, which are currently observed. Since the negative effect of PAL proteins in vaccines seems t be non-predictable, it would be advisable to avoid PAL proteins in vaccines unless specific evidence for a positive effect on protection is found. The existence of such negatively acting components gives further support to the need for development of well defined subunit vaccines against bacterial infections.

Acknowledgements

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References

[1] Taylor DJ. *Actinobacillus pleuropneumoniae*. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, editors. Diseases of swine. Ames, Iowa USA: Iowa State University Press; 1999. p. 343–54.

[2] Blackall PJ, Klausen HBLM, van den Bosch H, Kuhnert P, Frey J. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet Microbiol 2002;84:47–52.

[3] Frey J. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends Microbiol 1995;3(7):257–61.

[4] Schaller A, Kuhn R, Kuhnert P, Nicolet J, Anderson TJ, MacInnes JI, et al. Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology 1999;145:2105–16.

[5] Schaller A, Djordjevic SP, Eamens GJ, Forbes WA, Kuhn R, Kuhnert P, et al. Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene *apxIVA*. Vet Microbiol 2001;79(1):47–62.

[6] Kobisch M, van den Bosch JF. Efficacy of an *Actinobacillus pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society Congress, The Hague, The Netherlands, vol. 12; 1992. p. 216.

[7] van den Bosch JF, Pennings AMMA, Cuiljpers MECM, Pubben ANB, van Vugt FGA, van der Linden MFI. Heterologous protection induced by an *A. pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society Congress, Lausanne, Switzerland, vol. 11; 1990. p. 11.

[8] Martelli P, Guadagnini PF, Foccoli E, Ballarini G. Efficacy of an *Actinobacillus pleuropneumoniae* subunit vaccine in the control of pleuropneumonia: a field trial. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Bologna, Italy; 1996. p. 214.

[9] Pommier P, Ridremont B, Wessel-Roberts S, Keïta A. Field study into efficacy of a new *Actinobacillus pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Bologna, Italy; 1996. p. 206.

[10] Valks MMH, Nell T, van den Bosch JF. A clinical field trial in finishing pigs to evaluate the efficacy of a new APP subunit vaccine. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Italy; 1996. p. 208.

[11] Frey J, Kuhnert P, Villiger L, Nicolet J. Cloning and characterization of an *Actinobacillus pleuropneumoniae* outer membrane protein belonging to the family of PAL lipoproteins. Res Microbiol 1996;147(5):351–61.

[12] Rapp VJ, Ross RF. Antibody response of swine to outer membrane components of *Haemophilus pleuropneumoniae* during infection. Infect Immun 1986;54:751–60.

[13] Deich RA, Metcalf BJ, Finn CW, Farley JE, Green BA. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J Bacteriol 1988;170:489–98.

[14] Nelson MB, Apicella MA, Murphy TF, Vankeulen H, Spotila LD, Rekosh D. Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. Infect Immun 1988;56:128–34.

[15] Kasten RW, Hansen LM, Hinojoza J, Bieber D, Ruehl WW, Hirsh DC. *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *Haemophilus influenzae*. Infect Immun 1995;63:989–93.

[16] Green BA, Metcalf BJ, Quinn Dey T, Kirkley DH, Quataert SA, Deich RA. A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both nontypeable and type b *H. influenzae*. Infect Immun 1990;58:3272–8.

[17] Munson Jr RS. *Haemophilus influenzae*: surface antigens and aspects of virulence. Can J Vet Res 1990;54(Suppl):S63–7.

[18] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in molecular biology. New York, NY: Wiley; 1999.

[19] Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 1989;8:151–6.

[20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[21] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[22] Frey J, Nicolet J. Regulation of hemolysin expression in *Actinobacillus pleuropneumoniae* serotype 1 by Ca^{2+}. Infect Immun 1988;56:2570–5.

[23] Sebunya TN, Saunders JR, Osborne AD. A model aerosol exposure system for induction of porcine *Haemophilus pleuropneumonia*. Can J Comp Med 1983;47:48–53.

[24] Nielsen R, van den Bosch JF, Plambeck T, Sorensen V, Nielsen JP. Evaluation of an indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to the Apx toxins of *Actinobacillus pleuropneumoniae*. Vet Microbiol 2000;71(1–2):81–7.

[25] Yamanaka N, Faden H. Antibody response to outer membrane protein of nontypeable *Haemophilus influenzae* in otitis-prone children. J Pediatr 1993;122:212–8.

[26] Yamanaka N, Faden H. Local antibody response to P6 of nontypable *Haemophilus influenzae* in otitis-prone and normal children. Acta Oto-Laryngol 1993;113:524–9.

[27] Engleberg NC, Howe DC, Rogers JE, Arroyo J, Eisenstein BI. Characterization of a *Legionella pneumophila* gene encoding a lipoprotein antigen. Mol Microbiol 1991;5:2021–9.

[28] Burnens A, Stucki U, Nicolet J, Frey J. Identification and characterization of an immunogenic outer membrane protein of *Campylobacter jejuni*. J Clin Microbiol 1995;33:2826–32.

[29] Tibor A, Weynants V, Denoel P, Lichtfouse B, De Bolle X, Saman E, et al. Molecular cloning, nucleotide sequence, and occurrence of a 16.5-kilodalton outer membrane protein of *Brucella abortus* with similarity to pal lipoproteins. Infect Immun 1994;62:3633–9.

[30] Bouveret E, Benedetti H, Rigal A, Loret E, Lazdunski C. In vitro characterization of peptidoglycan-associated lipoprotein (PAL)-peptidoglycan and PAL-TolB interactions. J Bacteriol 1999;181(20):6306–11.

[31] Fortney KR, Young RS, Bauer ME, Katz BP, Hood AF, Munson Jr RS, et al. Expression of peptidoglycan-associated lipoprotein is required for virulence in the human model of *Haemophilus ducreyi* infection. Infect Immun 2000;68(11):6441–8.

[32] Munson Jr RS, Granoff DM. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect Immun 1985;49(3):544–9.

[33] Murphy TF, Bartos LC, Rice PA, Nelson MB, Dudas KC, Apicella MA. Identification of a 16,600-dalton outer membrane protein on nontypeable *Haemophilus influenzae* as a target for human serum bactericidal antibody. J Clin Invest 1986;78(4):1020–7.

[34] Kasten RW, Wakenell PS, Ahmad S, Yilma TD, Hirsh DC. Lack of protection against avian cholera by vaccination with recombinant P6-like protein from *Pasteurella multocida*. Avian Dis 1997;41(4):972–6.

PATENT Docket No.: 1038-1160

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Loosmore, et al.
Application No.:		09/857,843
Filing Date	:	September 17, 2001
Title	:	Multi-Component Vaccine Comprising At Least Two Antigens From Haemophilus influenzae To Protect Against Disease
Grp./AU	:	1645
Examiner	:	Jana A. Hines

November 26, 2003

APPEAL BRIEF AND REQUEST FOR EXTENSION OF TIME
TOTAL PAGES 75

BY FACSIMILE **703-872-9307**

The Commissioner of Patents and Trademarks
BOX AF,
Washington, D.C.
20231, U.S.A.

Dear Sir:

Introduction

This Appeal Brief is submitted pursuant to applicant's appeal from a Final Rejection of claims 1-5, 25 and 27 dated December 31, 2002. A Notice of Appeal was filed on June 3, 2003. The enclosed Credit Card Payment Form includes the prescribed fee. In the event of underpayment or overpayment please apply any additional charges or refunds to USPTO Deposit Account Number 500715. Three copies of the Appeal Brief are provided herewith.

Extension of Time

Petition is hereby made under the provisions of 37 CFR 1.136(a) for an extension of four months of the period for filing this Appeal Brief. The enclosed Credit Card Payment

Form includes the prescribed fee. In the event of underpayment or overpayment please apply any additional charges or refunds to USPTO Deposit Account Number 500715.

(1) **Real Party of Interest**

The real party of interest with respect to this patent application is Aventis Pasteur Limited. Assignments from the inventors to Aventis Pasteur Limited are recorded at Reel 013317/0711, 0724 and 0733 on September 20, 2002.

(2) **Related Appeals and Interferences**

The appellants, the appellants' legal representatives and assignee, are unaware of any pending appeals or interferences which will directly affect or be affected by or have a bearing on the Board's decision in the pending appeal.

(3) **Status of Claims**

This application was filed with claims 1-26. In the response dated September 25, 2002 to the Office Action of March 27, 2002 claims 6-24 and 26 were cancelled, claim 25 amended, and new claim 27 added.

Claims 1-5, 25 and 27 were finally rejected in an Office Action dated December 31, 2002. Claims 1-5, 25 and 27 are pending and the subject of this appeal and appear in Appendix I hereto.

(4) **Status of Amendments**

This application was filed with claims 1-26. Claims 1-5, 25 and 27 are pending and no amendments were filed subsequent to this final rejection.

(5) **Summary of Invention**

The present invention is directed to an immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae,* including otitis media. The composition comprises at least two different antigens of *Haemophilus influenzae,* at least one of which antigens is an adhesin (claim 1) and wherein said adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae,* (claim 2),

particularly an HMW 1 or HMW 2 protein of the non-typeable strain (claim 3), and said antigen which is not an adhesin is a non-proteolytic heat shock protein of a strain of Haemophilus influenzae (claim 4) and wherein the heat shock protein is an analog of *Haemophilus influenzae* Hin47 protein having a protease activity which is less than about 10% of that of the natural Hin47 protein (claim 5). The invention is further directed to compositions where the HMW protein is recombinantly produced and said antigen which is not an adhesin is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of the natural Hin47 protein in which the Histidine at amino acid position 91 is replaced by Alanine (claim 27). The present invention is further directed to a method of immunizing a host against disease caused by infection with *H. influenzae* (claim 25).

(6) Issues

The issues for consideration is the rejection of, claims 1-5 and 25 under 35 U.S.C. 112 1st paragraph and claims 1-5, 25 and 27 under U.S.C. 103(a) as being unpatentable over Barenkamp et al in view of Loosmore et al.

(7) Grouping of Claims

All claims do not stand or fall together, but rather each claim is individually patentable.

(8) Argument

(a) Background to the Invention

Haemophilus influenzae is the cause of several serious human diseases, such as meningitis, epiglottitis, septicemia and otitis media. There are six serotypes of *H. influenzae,* designated a to f, that are identified by their capsular polysaccharide. *H. influenzae* type b (Hib) was a major cause of bacterial meningitis until the introduction of several Hib conjugate vaccines in the 1980's. Vaccines based upon *H. influenzae* type b capsular polysaccharide conjugated to diphtheria toxoid, tetanus toxoid, or *Neisseria meningitidis* outer membrane protein have been effective in reducing *H. influenzae* type b-induced meningitis. The other serotypes of *H. influenzae* are associated with invasive disease at low frequencies, although there appears to be an increase in the incidence of disease caused by these strains as the incidence of Hib disease

declines. Non-encapsulated or non-typeable *H. influenzae* (NTHi) are also responsible for a wide range of human diseases including otitis media, epiglottitis, pneumonia and tracheobronchitis. The incidence of NTHi induced disease has not been affected by the introduction of the Hib vaccines.

Otitis media is the most common illness of early childhood, with 60 to 70% of all children, of less than 2 years of age, experiencing between one and three ear infections. Chronic otitis media is responsible for hearing, speech and cognitive impairments in children. *H. influenzae* infections account for about 30% of the cases of acute otitis media and about 60% of chronic otitis media. In the United States alone, treatment of otitis media costs between 1 and 2 billion dollars per year for antibiotics and surgical procedures, such as tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. It is estimated that an additional $30 billion is spent per annum on adjunct therapies, such as speech therapy and special education classes. Furthermore, many of the causative organisms of otitis media are becoming resistant to antibiotic treatment. An effective prophylactic vaccine against otitis media is thus desirable.

(b) The Present Invention

Having regard to the above Background, it would be desirable to provide efficacious combination vaccines comprising *H. influenzae* components containing selected relative amounts of selected antigens. The present invention provides an immunogenic composition for conferring protection in a host against disease caused by infection with *H. influenzae,* including otitis media.

The immunogenic composition comprises at least two different antigens of *H. influenzae,* one of which is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae* at least one of which is an adhesin as claimed in claim 1, and all claims dependant thereon.

Claim 2 recites that the adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *H. influenzae*. Claim 3 recites that the HMW protein is a HMW 1 or HMW 2 protein of a non-typeable strain of *H. influenzae*. Claim 4 recites that the antigen which is not an adhesin is a non-proteolytic heat shock protein of a non-typeable strain of *H. influenzae*. Claim 5 recites that the heat shock protein is an analog of *Haemophilus influenzae* Hin47 protein having a protease activity which is less than about 10% of that of the natural Hin47 protein.

Claim 27 further recites that the Hin47 protein having a decreased protease activity which is less than about 10% of that of the natural Hin47 protein in which the Histidine at amino acid position 91 is replaced by Alanine. The applicants data supports such results.

(c) Rejection of claims 1-5 and 25 under 35 U.S.C. 112 1st paragraph.

The HMW proteins are described in Barenkamp et al cited by the Examiner in the prior art rejection while the Hin47 analogs are described in the Loosmore et al reference cited by the Examiner in the prior art rejection. In the Barenkamp et al reference, there is described both the isolation of the HMW protein from natural-source materials and recombinant production. It is submitted, that the enablement is not limited to recombinantly-produced HMW protein.

In the Loosmore et al reference, there is described the manner of producing the non-proteolytic analog of Hin47 protein. In this respect, at least one amino acid contributing to protease activity is deleted or replaced by a different amino acid. The Loosmore et al reference describes how to identify such amino acid by comparison to known proteases. The reference specifically describes that the deleted or replaced amino acid may be selected from amino acids 195 to 201 and specifically describes replacement of Serine-197 with alanine, other specific amino acid mutations described are Histidine-91 replaced with alanine, and lysine or arginine-121 replaced with alanine. The immunogenic properties of these various mutants are described in Loosmore et al. Based on this information, there is no reason to suppose that any other non-proteolytic analog would not also function in the same manner as the specific H91A Hin47 analog utilized in the experiments described in the application (page 18 lines 4-12). It is submitted that enablement is not limited to the specific H91A Hin47 analog, but rather extends at least to any non-proteolytic analog of the Hin47 protein. The Examiner indicates that the objection of lack of enablement is based, to some extent, upon lack of guidance as to how to determine compositions other than that specifically identified by the Examiner. It is submitted that such is not the case.

Specifically, the specification tells a person skilled in the art that two different antigens of *Haemophilus influenzae* are employed and that one of them has to be an adhesin and the other does not. Testing to determine if an antigen is an adhesin or not an adhesin is within the skill of the art. In this regard, the Examiner's attention is directed to the experimentation described in Barenkamp.

In addition, the person skilled in the art is advised that one such adhesin protein is the HMW protein, where that is described and how to produce it both from natural-source materials and recombinantly (see page 2, line 23 to page 3, line 23). In addition, the person skilled in the art is advised that one such nonadhesin protein is a non-proteolytic analog of Hin47 protein or other non-proteolytic heat shock protein and how to produce such an analog (see page 3, line 14 to page 3 line 31).

Furthermore applicants have given guidance to one skilled in the art to test if a composition is an immunogenic composition (see example 4 pages 18 to 20 of the present application).

Having regard to the foregoing discussion, it is submitted that claims 1 to 5 and 25 are fully enabled by the disclosure.

(d) Rejection of claims 6-24 under 35 USC 103(a).

Claims 1 to 5, 25 and 27 have been finally rejected under 35 USC 103(a) as being unpatentable over Barenkamp (WO 97/36914) in view of Loosmore et al (US Patent 5,506,139).

Claim 1 defines an immunogenic composition for conferring protection in a host against disease caused by *Haemophllus influenzae* comprising at least two different antigens of *Haemophilus influenzae.*

- one of which antigens is an adhesin
- the other of which antigens is not an adhesin.

The Examiner has identified Barenkamp et al as describing a *Haemophilus influenzae* protein which is an adhesin and Loosmore as describing a *Haemophllus influenzae* protein which is not an adhesin. The applicants position is that neither reference provides the motivation to combine the two immunogens in a single composition, as required by claim 1.

Barenkamp teaches high molecular weight proteins of non-typeable *H. influenzae* identified as HMW1, HMW2, HMW3 and HMW4, which are characterized by molecular weight and sequence information. Loosmore et al teach an analog of *H. influenzae* Hin47 protein with reduced protease activity. It is submitted that these references lack any motivation to combine two different antigens of *H. influenzae,* namely a non-proteolytic Hin47 protein of Loosmore et

al with the HMW proteins of Barenkamp et al in an immunogenic composition. It is the applicants position that neither reference provides the motivation to combine the two immunogens in a single composition as required by claim 1, and by dependency all claims on appeal.

While suggesting various combinations, there is no suggestion here to combine different proteins derived from the same pathogen, as in applicants claim 1. Again, the references are silent as to any specific combination contemplated.

The cited prior art lacks the motivation to do so. There are vague, non-specified indications in both references to combine other components with the specific immunogen, but there is no specific indication as to what that other component may comprise, other than an adjuvant or materials from the pathogens and/or materials from various strains of the same pathogen.

As the Examiner has pointed out, on page 49, lines 15 to 19 of Barenkamp, it is stated:

> "…. the data suggests the HMW adhesin proteins are potentially important protective antigens which may comprise one component of a multi-component NTHI vaccine."

This passage appears to suggest that only *Haemophilus* proteins which are the HMW adhesin proteins are appropriate components. The non-proteolytic analog of Hin47 is not an adhesin (although initially thought to be adhesin, see col. 2, line 17 of Loosmore et al). (It is pointed out that the Examiner is incorrect in the statement that the adhesin protein "should" comprises one component of the NTHI vaccine. As can be seen from the above quotation, Barenkamp uses the word "may").

Even if the Examiner finds motivation in this passage of Barenkamp to combine the HMW protein with another *Haemophilus* antigen, whether an adhesin or not, such motivation still provides no motivation to select the non-proteolytic Hin47 analog as the other *Haemophilus* antigen.

There have been a significant number of *Haemophilus* proteins identified as vaccine candidates besides the HMW and Hin47 analog proteins. These proteins include the

various outer membrane proteins A to H, lactoferrin and transferrin receptor protein and the P1, P2, P6 and D15 proteins. It is submitted that there is no motivation provided by the cited prior art why a person skilled in the art would specifically select from all the optional possibilities, the non-proteolytic Hin47 analog to specifically combine with the HMW protein.

The Examiner states in the Office Action, quoting In re Kerkhoven, that:

> "The idea of combining them flows logically from their having been individually taught in the prior art."

The "idea of combining them" does not explain why the two materials should be combined when there is selection available. If the two antigens were the only two known antigens of *Haemophilus influenzae*, then there may be some validity to the position taken by the Examiner, but this is clearly not the case here.

In any event, caution is required when considering combining different antigens into immunogenic compositions because of the danger of impairment of the immunogenicity of the individual components one by the other. As may be seen from Applicants data, in Figure 3, immunogenic compositions are provide in which there is no impairment of individual antigenic components.

Furthermore, these results are unexpected in the field of combination vaccines. There is little expectation of success that simply mixing existing vaccine antigens will not result in incompatibilities amongst the various antigens, resulting in loss of stability or reduced potency or indeed a synergistic effect increasing potency. Immune interference cannot be predicted. Others skilled in the art of combination vaccines have found that the preparation of combination vaccines is far from straight forward. For example Cauldfield et al (2001) report on the need for a balanced formulations of vaccine components in the preparation of DTP combination vaccines to circumvent interference with the components. Van den Bosch et al (2003) have also reported that the addition of a potential antigen (Pal A) from *Actinobacillus pleuropneumoniae* can completely eliminate the positive efficacy of known antigens (ApxI and II) when combined (see abstract).

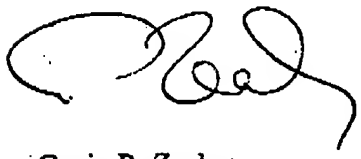
For all these reasons, it is submitted that claims 1 to 5, 25 and 27 are patentable over the applied art and the rejection thereof under 35 USC 103(a) as being unpatentable over

Barenkamp in view of Loosmore et al. cannot be sustained.

Summary

Having regard to the above detailed discussion, it is submitted that the Examiner is in error in rejecting claim 1 to 5, 25 and 27 as being unpatentable under 35 USC 112 1st paragraph and the rejection under 35 USC 103(a) as being unpatentable over the combination of Barenkamp in view of Loosmore et al, should be REVERSED.

Respectfully submitted,



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(9) **APPENDIX I**

CLAIMS APPEALED (09/857,843)

1. An immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae,* comprising:
at least two different antigens of *Haemophilus influenzae,* at least one of which antigens is an adhesin.

2. The immunogenic composition of claim 1 wherein said antigen which is an adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae.*

3. The immunogenic composition of claim 2 wherein said HMW protein is a HMW 1 or HMW2 protein of the non-typeable strain of *Haemophilus influenzae.*

4. The immunogenic composition of claim 1 wherein the antigen of *Haemophilus influenzae* which is not an adhesin is a non-proteolytic heat shock protein of a strain of *Haemophilus influenzae.*

5. The immunogenic composition of claim 4 wherein the non-proteolytic heat shock protein of a strain of *Haemophilus influenzae* is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein.

25. A method of immunizing a host against disease caused by infection with *Haemophilus influenzae,* including otitis media, which comprises administering to the host an immunoeffective amount of a composition as claimed in claim 1.

27. The composition of claim 1 wherein said antigen which is an adhesin is a recombinantly-produced high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus*

influenzae and said antigen which his not an adhesin is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein, in which Histidine-91 is replaced by alanine.

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Immunogenicity of a hexavalent combination vaccine in rhesus monkeys

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Abstract

Preclinical immunogenicity studies were conducted in rhesus monkeys to determine whether there is immune interference in the response to one or more components of a hexavalent vaccine (Hexavac™) that contains antigens from *Haemophilus influenzae* (Hib), hepatitis B (HB), diphtheria (D), tetanus (T), acellular pertussis (aP) and inactivated polio virus (IPV). Antibody responses were measured following co-administration of the components at three separate anatomical sites or administration as a hexavalent combination in a single site. After three injections of the hexavalent vaccine, the peak antibody responses to each component of the vaccine were >100-fold above pre-immune titers and persisted at levels >10-fold above pre-immune titers at ≈1 year. Immune interference was observed in the peak response to HB, D and pertussis toxin, but was not seen at later time points. The results indicate that the rhesus monkey model may be useful for pre-clinical evaluation of combination vaccines.

Keywords: Combination vaccine; Immune interference; Antigen competition; Non-human primate

1. Introduction

The rationale for the development of combination vaccines has been discussed in recent publications [1–3]. The main benefits are to enhance compliance and vaccine coverage and to reduce overall healthcare costs. An added benefit is to 'make room' in the pediatric vaccination schedule for new vaccines projected for the new millennium [4]. However, experience has shown that the preparation of combination vaccines is far from straightforward. During the development of the DTP combination vaccine, the need for 'balanced' formulations of vaccine components was recognized [5] and careful dose-ranging of the three serotypes of the oral poliovirus vaccine (OPV) was required in order to circumvent interference of the type 2 strain on the immune response to types 1 and 3 [6]. Th main impediments to the development of combination vaccines are stability and immunogenicity. Simply mixing existing vaccines can result in incompatibilities among the various antigens, adjuvants, preservatives, stabilizers and excipients, resulting in a loss of stability or reduced potency [7]. A further confounding factor is that of immune interference (also known as antigen competition) which may not always be predicted using animal models. Antigenic competition was first described by Michaelis in 1904 [8], but is still poorly understood.

The objective of the present preclinical immunogenicity studies of Hexavac™ was to determine the antibody response to vaccine component antigens at various times after immunization and to compare the response to the hexavalent vaccine with that induced by administration of Hib, HB and DTaP-IPV at separate anatomic sites. The results indicat that there was a significant difference between experimental and control arms in the peak responses to HB, D and PT. These differences fade with time and there was no significant

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difference in the response to any vaccine component tested at 48–51 weeks post-dose 1. Overall, strong antibody responses were induced to each component of Hexavac™ in rhesus monkeys.

2. Materials and methods

2.1. Experimental animals

The rhesus monkeys (*Macaca mulata*) used in this study were born at the California Regional Primate Center at the University of California at Davis and all immunizations and blood collection procedures were performed at that site. Some monkeys were housed outdoors in social groups, whereas others were maintained indoors, in pairs. Those maintained indoors had a 12:12 h light:dark cycle within a temperature range of ≈17–29°C. Animals were all fed Purina Monkey Chow, 15% protein with fresh produce supplements two to three times per week. Monkeys were identified by tattoos containing unique numbers. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Vaccine composition

The hexavalent vaccine consisted of Hib capsular polysaccharide (polyribosyl ribitol phosphate) conjugated to tetanus toxoid (PRP-T), hepatitis B (HB), surface antigen (HBsAg), diphtheria toxoid (D), tetanus toxoid (T), pertussis filamentous hemagglutinin (FHA), pertussis toxoid (PT) and three serotypes of inactivated poliovirus (IPV) formulated with aluminum adjuvants. Each 0.5 ml dose contained 12 μg PRP-T (expressed in polyoside), 5 μg HBsAg, 30 Lf D, 10 Lf T, 25 μg FHA, 25 μg PT, IPV type 1 (40 D-Ag U), type 2 (8 D-Ag U) and type 3 (32 D-Ag U).

2.3. Vaccination/schedule

One group of rhesus monkeys was immunized with half of the human pediatric dose of the hexavalent combination vaccine (Hexavac™) into a single i.m. site, while a second cohort (control group) of monkeys was injected with half of the human dose of PRP-T (ActHIB®), HBsAg (RECOMBIVAX HB®) and DTacP-IPV at three separate i.m. sites at 0, 4 and 8 weeks, according to the protocol shown in Table 1. The monkeys were weighed at each time point and examined for injection site reactions after each dose of vaccine. In addition, blood samples collected at each time point were monitored for changes in white cell, red cell and platelet levels.

2.4. Serological assays

Sera were collected at week 0, 4, 8, 10 and 48–51 and tested individually for antibody titers against PRP, HBsAg, D, T, FHA and PT. Due to a shortage of sera, serology was not performed to detect antibodies against poliovirus. Anti-PRP (component of Hib) antibody titers were measured using a Farr-type radioimmunoassay (RIA), as previously described [9,10]; responses to HB were determined using a modified Ausab® assay (Abbott Laboratories, N. Chicago, IL), as described elsewhere [11]. Antibodies against FHA [12] and antibody titers against T were measured by ELISA [13]. Diphtheria toxoid antibody titers were assayed by using a neutralization test in comparison to a WHO antitoxin standard [14]. PT antibody titers were also determined by a toxin neutralization test on CHO cell culture [15]. The results are expressed as geometric means. The assays used were originally validated for analysis of human samples and adapted for testing monkey serum without further analytical validation.

2.5. Statistical analysis

At each time point, the estimated GMT ratio for the experimental vaccine group relative to the control group (Hexavac™/Hib + HB + DTaP-IPV) and corresponding two-sample 99% confidence interval for the true GMT ratio are calculated for the response to each antigen; assuming unknown but equal variances between the two groups. If a particular interval excludes

Table 1
Protocol for preclinical immunogenicity testing of Hexavac™ in rhesus monkeys

Group	n	Age at dose 1 (months)	Vaccine	Injection schedule (weeks)	Bleeding schedule (weeks)
1	8[c]	6–12	Hexavac™[a]	0, 4, 8	0, 4, 8, 10, ≈50
2	8	4.8–10.5	ActHIB® + RECOMBIVAX HB® + DTaP-IPV[b]	0, 4, 8	0, 4, 8, 10, ≈50

[a] Injection volume of 0.25 ml in one intramuscular site.
[b] Injection in three separate intramuscular sites (0.25 ml each).
[c] Serum samples from six of eight monkeys were available at the week ≈50 time point.

the value 1, the corresponding comparison between Hexavac™ and Hib + HB + DTaP-IPV is statistically significant; otherwise, it is not. The reason for using a 99% confidence level instead of the usual 95% level is to control the overall false–positive rate (per antigen), which is defined as the probability that at least one of the confidence intervals will exclude the value 1 by chance alone [16].

3. Results

3.1. Serum antibody response to vaccination

Antibody titers to each component of the vaccine (except IPV) were measured at week 0, 4, 8, 10 and 48–50 using sera from individual animals (Fig. 1). At week 4 (post-dose 1), there were no significant differences between groups in antibody titers to any of the antigens tested. Similarly, at week 8 (4 weeks post-dose 2), there were no significant differences among groups with the exception of the response to HB, which was significantly higher in the control group compared with the monkeys injected with the hexavalent combination. As shown in Table 2, at the 10-week time point (2 weeks post-dose 3), there was a significantly higher response to three of the vaccine components (HB, D and pertussis toxin) in monkeys immunized with separate injections of Hib + HB + DTaP-IPV compared with the response of monkeys immunized with the hexavalent combination vaccine. Importantly, at the final time point (week 48–51), which is 38–41 weeks post-dose 3, there was no significant difference in the response to any component of the vaccines.

3.2. Response rate to vaccination

The percentage of responders to components of the vaccine was determined at each bleed time point. In the absence of established 'seroprotective titers' for rhesus monkeys, the accepted human equivalents were used as shown in the legend to Table 3. For pertussis, there is no proven correlate of protection established for humans, therefore, the percentage of seroconverters was used instead. As shown in Table 3, there was no difference in the response rate to vaccination with Hexavac™ compared with separate site administration of Hib + HB + DTaP-IPV except for the response to HB at the 8-week time point. At that time, only 37% (3/8) monkeys responded to Hexavac™ whereas 100% (8/8) responded to the control. These results are consistent with the analysis of the serological titers that showed a significant difference in anti-HBs titers at this time.

3.3. Adverse event monitoring

Animals were monitored for changes in weight or blood cell counts as well as for injection site reactions. No adverse reactions were noted at the site of injection at any time point and there was no adverse effect of vaccination on the weight or blood cell counts of any animals (data not shown).

4. Discussion/conclusion

The results from the present pre-clinical evaluation of Hexavac™ indicate that there was a vigorous response to each component of the vaccine. Even so, there was evidence for interference in the peak response to HB, D and pertussis toxoid when the responses to Hexavac™ were compared with the control group. Four types of immune interference (antigen competition) have been described: (a) sequential; (b) intramolecular; (c) intravirionic; and (d) intermolecular competition.

- Sequential competition occurs when a second antigen (or vaccine) is given shortly after a first antigen (or vaccine) [17]. This form of interference is especially relevant to vaccine dosing schedules.
- Intramolecular competition results from competition among peptides derived from the same protein for binding to MHC Class I or Class II molecules [18].
- Intravirionic competition results when one protein antigen within a virus interferes with the response to a second protein antigen within the same virus [19]. This form of antigen competition can be circumvented by dissociation of the virus into its component parts prior to immunization.
- Intermolecular competition results when one antigen in a mixture interferes with the immune response to a second antigen in a mixture [17,20]. This form of interference is most relevant to the present investigation; however, the mechanism by which this happens is unknown. One possibility is that one or more components within the combination vaccine become unstable, perhaps due to excipients carried over into the vaccine with a separate component. However, extensive stability studies have been performed on Hexavac™ and the components that had reduced immunogenicity in the combination vaccine (HB, D and PT) were shown to be stable for several years (data not shown). Thus, the decreased response to certain of the vaccine components of Hexavac™ does not appear to be related to a loss of stability of these components, suggesting that the explanation is immune interference due to intermolecular antigen competition.

Although the rhesus monkey model suggests that the response to Hexavac™ is marked by transient interference in response to the HB, D and PT components,

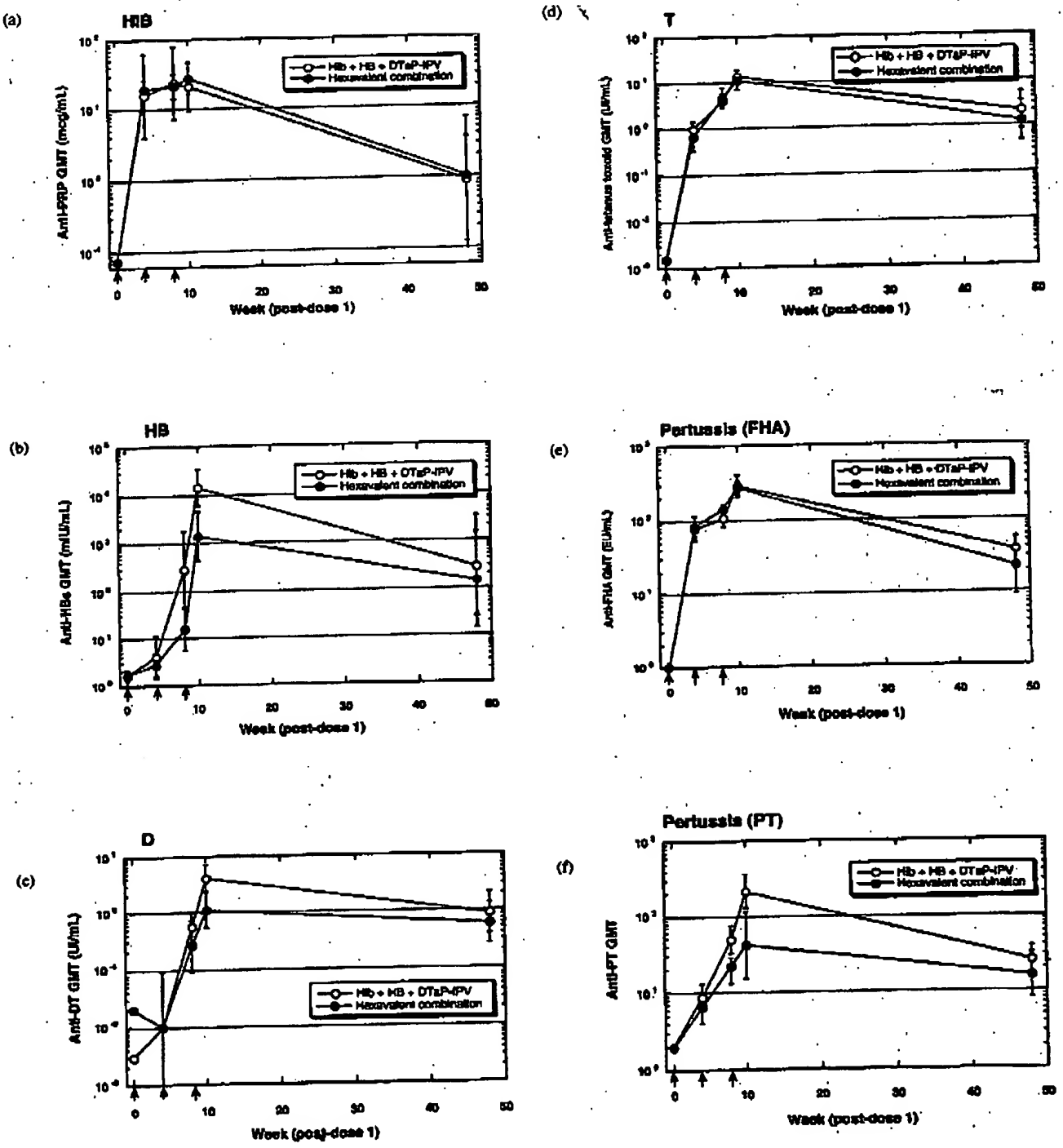

Fig. 1. Antibody response to antigens from Hib, HB, D, T, FHA and PT in rhesus monkeys immunized with Hib + HB + DTaP-IPV at separate sites or with the hexavalent combination vaccine (Hexavac™) at a single i.m. site. Monkeys were immunized at week 0, 4 and 8 and antibody titers were determined on serum collected at week 0, 4, 8 and ≈50. Results are expressed as the geometric mean with 95% confidence intervals.

these apparent differences in potency are unlikely to translate into clinically meaningful differences since: (a) the response to each component of the vaccine is > 100-fold higher than the pre-immune titers; (b) the response rate post-dose 3 (percent seroconverters) is equivalent in the two groups; and ( ) the difference in titers elicited by Hexavac™ and the control arm became indistinguishable over time. By study week 48–51,

Table 2
Estimated ratios (Hexavac™/Hib+HB+DTaP-IPV) and 99% confidence intervals for the antibody response to Hexavac™ versus control[a]

Antigen	Week (post-dose 1)			
	4	8	10	48–51
HB	0.69 (0.15, 3.21)	**0.06 (0, 0.8)**	**0.1 (0.02, 0.063)**	0.5 (0.01, 37.61)
Hib	1.21 (0.2, 7.16)	0.9 (0.23, 3.56)	0.76 (0.23, 2.56)	2.24 (0.14, 36.74)
D	1.49 (0.09, 25.11)	0.47 (0.1, 2.1)	**0.27 (0.08, 0.86)**	0.66 (0.14, 3.04)
T	0.69 (0.25, 1.95)	1.17 (0.55, 2.49)	0.83 (0.39, 1.79)	0.61 (0.11, 3.4)
FHA	1.08 (0.58, 2.03)	1.31 (0.88, 1.95)	0.94 (0.57, 1.57)	0.6 (0.2, 1.82)
PT	0.77 (0.38, 1.55)	0.46 (0.19, 1.09)	**0.19 (0.05, 0.82)**	0.65 (0.26, 1.64)

[a] Any confidence interval excluding the value '1' implies a statistically significant difference. If the upper confidence limit is <1, then the GMT of Hexavac™ is significantly less than the GMT of Hib+HB+DTaP-IPV. Statistically significant results are in bold type.

Table 3
Response rate to Hexavac™ versus the control group[a]

Antigen	Percent responders (Hexavac™, control) at week:				
	Pre	4	8	10	48–51
HB	0, 0	0, 12	37, 100	100, 100	83, 87
Hib	12, 12	100, 100	100, 100	100, 100	83, 75
D	33, 0	17, 0	100, 100	100, 100	100, 100
T	0, 0	0, 0	100, 100	100, 100	100, 100
FHA	0, 0	0, 0	100, 100	100, 100	100, 100
PT	0, 0	62, 87	100, 100	100, 100	100, 100

[a] Criteria for response: HB (>10 mIU/ml); Hib (>0.15 mcg/ml); D (>0.01 IU/ml); T (>0.01 IU/ml); FHA (>4 UE/ml); PT (reciprocal titer >4).

there was no significant difference in the antibody response to any component of the vaccine between monkeys injected with Hexavac™ versus separate site injection of Hib + HB + DTaP-IPV. The kinetics of the response also deserves comment. With the exception of the response to Hib, the titers to each vaccine component increased following each dose of vaccine. The reason for the immediate and vigorous response to Hib is not known, however, the rapid response suggests that the monkeys may have been primed by prior exposure to *Haemophilus influenzae* or to a cross-reacting organism. Subsequent injections of Hexavac™ did not significantly increase the high titers observed after the first injection, which were >10 μg/ml. In previous studies [10], 18–22-month-old rhesus monkeys were found to respond earlier and with higher titers to a Hib vaccine (PedvaxHIB®) than 2–3-month-old monkeys. This suggests that the use of younger monkeys may enable better discrimination among Hib-containing vaccines.

The response rate of rhesus monkeys that received three doses of the vaccine was 100% (at week 10). This compares favorably with the response rates seen in a clinical trial of Hexavac™ in which the response rate was 87–100% for each of the antigens [3]. However, the antibody titers achieved in rhesus monkeys were ≈10-fold higher that titers attained in th human post-dose 3. The magnitude of the response of the rhesus monkeys to three doses of Hexavac™ was similar to that of human infants given four injections of Hexavac™ [3]. It should be noted, however, that the small sample size ($n = 8$ monkeys per group) is a limitation in the interpretation of antibody titers and response rates. With these caveats, the rhesus monkey animal model described herein may be useful in the evaluation and development of future combination vaccines.

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References

[1] Ellis RW. Development of combination vaccines. Vaccine 1999;17:1635.
[2] Andre FE. Development and clinical application of new polyvalent combined paediatric vaccines. Vaccine 1999;17:1620.
[3] Pines E, Barrand M, Fabre P, Salomon H, Blondeau C, Wood SC, et al. New acellular pertussis-containing paediatric combined vaccines. Vaccine 1999;17:1650.

[4] Eskola J. Epidemiological views into possible components of paediatric combined vaccines in 2015. Biologicals 1994;22:323.
[5] Barr M, Llewellyn-Jones M. Some factors influencing the response to immunization with single and combined prophylactics. Br J Expti Pathol 1955;36:147.
[6] Plotkin S, Koprowski H, Stokes J. Clinical trials in infants of orally administrated attenuated poliomyelitis viruses. Pediatrics 1968;23:1041.
[7] Corbel MJ. Control testing of combined vaccines: a consideration of potential problems and approaches. Biologicals 1994;22:353.
[8] Michaelis L. Weitere untersuchungen uber eiweißprazipitine. Dtsch Med Wschr 1904;30:1240.
[9] Kuo J, Monji N, Schwalbe R, McCoy D. A radioactive antigen binding assay for the measurement for the measurement of antibody to haemophilus influenzae type b capsular polysaccharide. J Immunol Methods 1981;43:35.
[10] Vella P, Staub J, Armstrong J, Dolan K, Rusk C, Szymanski S, et al. Immunogenicity of a new *Haemophilus influenzae* type b conjugate vaccine (Meningococcal protein conjugate) (Pedvax-HIB®). Pediatrics 1990;85:S668.
[11] Wang S, Liu X, Fisher K, Smith JG, Chen F, Tobery TW, et al. Enhanced type I immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminum-phosphate. Vaccine 2000;18:1227.
[12] Manclark C, Meade B, Burstyn D. Serological response to Bordatella pertussis. In: Rose N, Friedman H, Fahey J, editors. Manual of Clinical Laboratory Immunology, 3rd ed. Washington, DC: American Society for Microbiology, 1986:388.
[13] Bizzini B. Tetanus. In: Germanier R, editor. Bacterial Vaccines. Orlando, FL: Academic Press, 1984:37.
[14] Miyamura K, Nishio S, Ito S, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using Vero cells: I. Studies on factors affecting the toxin in microplate culture Chinese Hamster Ovary cells. J Biol Stand 1974;2:189.
[15] Gillenius P, Jaatmaa E, Askelof P, Granstrom M, Tiru M. The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese Hamster Ovary cells. J Biol Stand 1985;13:61.
[16] Fisher LD, van Belle G. Biostatistics: A Methodology for the Health Sciences. New York: Wiley, 1983.
[17] Taussig MJ. Antigenic competition. Curr Topic Microbiol Immunol 1973;60:125.
[18] Babbit BP, Matwueda G, Haber E, Unanue ER, Allen PM. Antigenic competition at the level of peptide-Ia binding. Proc Natl Acad Sci USA 1986;83:4509.
[19] Johansson BE, Moran TM, Kilbourne ED. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. Proc Natl Acad Sci USA 1987;84:6869.
[20] Pross HF, Eidinger D. Antigen competition: a review of nonspecific antigen-induced suppression. Adv Immunol 1974;18:133.

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Interference of outer membrane protein PalA with protective immunity against *Actinobacillus pleuropneumoniae* infections in vaccinated pigs

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Abstract

The role of antibodies to the outer membrane protein PalA of *Actinobacillus pleuropneumoniae* in protective immunity was studied in pigs vaccinated with purified PalA alone and PalA in combination with toxoids of the RTX toxins ApxI and ApxII using an established challenge model with the virulent serotype 1 of *A. pleuropneumoniae*. Pigs that developed antibody titers against PalA after immunization were more significantly affected by challenge with *A. pleuropneumoniae* serotype 1. Following challenge, pigs that were immunized with PalA showed more severe respiratory symptoms, had a higher mortality rate and died faster. They also displayed much more severe lung lesions after necropsy than animals not immunized with PalA. Pigs that were immunized with toxoids of the two cytotoxins ApxI and ApxII were protected against challenge with *A. pleuropneumoniae*. In contrast, the protective efficacy of the ApxI and ApxII vaccine was completely lost when it was supplemented with PalA. Hence, antibodies induced against the outer membrane protein PalA of *A. pleuropneumoniae* aggravated the consequences of infection and counteracted the protective effect of anti-ApxI and anti-ApxII antibodies. Due to the high similarity between protein analogues of PalA from various bacteria of the *Pasteurellaceae* family such as P6 of *Haemophilus influenzae* or 16 kDa Omp of *Pasteurella multocida*, this deleterious effect of PalA in vaccination should be taken into consideration in the development of vaccines against infections with other *Pasteurellaceae*.

Keywords: Outer membrane protein; Experimental infection; Antigenicity; Immunity; Vaccine; Porcine pleuropneumonia; *Pasteurellaceae*

1. Introduction

Actinobacillus pleuropneumoniae, a Gram-negative bacterium of the family *Pasteurellaceae*, is the etiological agent of porcine pleuropneumonia, a severe contagious disease of swine with worldwide prevalence [1]. To date, fifteen serotypes have been described which variously express three different cytotoxins belonging to the RTX toxin family: ApxI, ApxII, ApxIII [2]. These toxins mainly determine the virulence of the different serotypes [3]. A fourth RTX-toxin, ApxIV, which is expressed by all serotypes of *A. pleuropneumoniae*, was recently detected and shown to be produced during infection but not during culture in growth medium [4,5]. Serotypes 1, 5a/b, 9 and 11, which express ApxI, ApxII and also ApxIV, are particularly virulent, while the other serotypes which are devoid of ApxI are generally less virulent [3]. Vaccination is an effective strategy for the prevention of porcine pleuropneumonia outbreaks. Recently, a new generation subunit vaccine, composed of the three major RTX exotoxins (ApxI, ApxII and ApxIII) and a 42 kDa outer membrane protein of *A. pleuropneumoniae*, has been developed and shown to give high protection against all 12 major serotypes (serotypes 1–12) under experimental conditions [6,7] as well as in field trials [8–10]. Vaccination of pigs with the RTX toxins alone protects against mortality but generally fails to reduce the typical *A. pleuropneumoniae* lung lesions, while the combination of RTX toxins with the 42 kDa outer membrane protein (42 kDa OMP) induced complete protection with regard to mortality as well as lung lesions [6]. The 42 kDa OMP is therefore a valuable component of the vaccine. Among the OMPs of *A. pleuropneumoniae*, PalA is the most immuno-predominant antigen [11,12]. PalA is a 14 kDa protein, encoded by *palA* as a precursor peptide which is processed by signal sequence peptidase II and sorted by a peptide located signal

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for the final localization of the mature protein in the outer membrane. PalA is conserved in all *A. pleuropneumoniae* serotypes and induces a strong IgG response in pigs naturally or experimentally infected with *A. pleuropneumoniae* [11]. PalA shows high amino-acid (aa) sequence homology to the family of peptidoglycan associated proteins (PAL) of Gram-negative bacteria showing most similarity to the P6 protein of *Haemophilus influenzae* [11,13,14] and to the 16 kDa Omp of *Pasteurella multocida* [15]. There is evidence that PAL proteins of *Pasteurellaceae*, in particular P6 f *H. influenzae*, can act as protective antigens. P6 was therefore suggested to be included in vaccines against *H. influenzae* induced meningitis and avian cholera [15,16]. Antiserum directed against recombinant P6 protein was shown to be bactericidal against clinical *H. influenzae* isolates, including highly pathogenic non-typable strains [17]. Antibodies to P6 give passive protection to infant rats against *H. influenzae* type b-induced meningitis. In the view of the importance of PAL proteins as potential vaccine antigens, we have designed the present study to analyze the role of PalA alone, and in combination with toxoids of the RTX toxins ApxI and ApxII in induction of protective immunity against challenge of pigs with a virulent *A. pleuropneumoniae* serotype 1 strain, 4074^T.

2. Materials and methods

2.1. Bacterial strains, growth conditions and vectors

A. pleuropneumoniae 4074^T (serotype 1 reference strain) used for PCR amplification of the *palA* gene, and *A. pleuropneumoniae* serotype 1 strain 1-L-452 used for challenge, were grown on solid Columbia broth agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10 mM β-NAD (Sigma Chemicals, St. Louis, MO, USA) or in liquid medium consisting of Columbia broth supplemented with 10 mM β-NAD. In order to avoid appearance of contaminating bacteria during re-isolation of *A. pleuropneumoniae* from the challenged pigs, lungs and tonsils were first superficially decontaminated with a hot spatula before sampling with sterile tools from the inside of the tissues.

Escherichia coli K-12 strain XL1-blue MRF' (Stratagene, La Jolla, CA, USA) and *E. coli* B strain BL21 (DE3) (Novagen, Madison, WI, USA) were grown in Luria-Bertani (LB) broth [18] at 37 °C in orbital shaker incubator. Ampicillin 100 μg/ml was added when needed for selection or stabilization of plasmids. Cloning vector pETHIS-1 [4] was used for the production of recombinant poly-histidine tailed peptides.

2.2. DNA extraction, manipulation, cloning and sequence analysis

Gen mic DNA from *A. pleuropneumoniae* was extracted by the guanidiumthiocyanate method [19]. Ligation, gene cloning, plasmid extraction, restriction endonuclease digestion and analysis of the DNA fragments by agarose gel electrophoresis were performed using standard protocols [18]. Plasmid extraction was done using the alkaline lysis method with the Miniprep kit (Qiagen AG, Basel, Switzerland). DNA sequencing reactions were performed with approximately 500 ng plasmid DNA per reaction mixture and 5 pmol of primer. Sequences were determined with an ABI Prism model 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled and edited by using the Sequencher 3.0 program (Gene Codes Corp., Ann Arbor, MI, USA) to obtain contiguous sequences.

2.3. Production of antigens for vaccines

PalA protein from *A. pleuropneumoniae* serotype 2 strain S411 for vaccination was produced as described earlier and involved heat extraction in PBS buffer (Na-phosphate 50 mM, pH 7.5, NaCl 0.85%) for 1 h at 60 °C, ammonium sulfate fractionation and separation by SDS-PAGE followed by electroelution [11]. The commercially available subunit vaccine, Porcilis App™ (Intervet International, Boxmeer, the Netherlands), was used as reference preparation for the titration of the ApxI and ApxII antigens. ApxI and ApxII proteins were prepared from supernatants of *A. pleuropneumoniae* serotype 5b bacteria grown at 37 °C for 6 h in Columbia broth supplemented with βNAD 10 μg/ml and $CaCl_2$ 25 mM. Cells were removed by centrifugation and sterile filtration through a low protein binding 0.45 μm filter (Acrocap, #4482, Gelman Laboratory, Ann Arbor, MI, USA). ApxI and ApxII were subsequently concentrated by ultrafiltration on a 500 kDa MW cut-off polyether sulfon filter (Amicon, bioseparations, Millipore, Bedford, MA, USA). Protein concentrations were measured by the method of Bradford [20]. ApxI and ApxII preparations were analyzed by standard SDS-PAGE stained with Coomassie blue [21] where they revealed a predominant band at 105 kDa indicating that ApxI and ApxII represented the major proteins (estimated to 90% of the total proteins) in the preparations [22].

For the vaccination of pigs, the following vaccines were produced: (i) Vaccine I (PalA) consisted of 80 μg PalA protein in 2 ml Diluvac Forte® adjuvant formulation (Intervet International) per vaccine dose with 0.02% (v/v) formaldehyde final concentration. (ii) Vaccine II (PalA + ApxI + ApxII) consisted of 80 μg recombinant PalA, ApxI and ApxII in 2 ml Diluvac Forte® adjuvant formulation per vaccine dose with 0.02% (v/v) formaldehyde final concentration. (iii) Vaccine III (ApxI + ApxII) consisted of ApxI and ApxII in 2 ml Diluvac Forte® adjuvant formulation per vaccine dose with 0.02% (v/v) formaldehyde final concentration; the concentrations of ApxI and ApxII in vaccines II and III were titrated by specific antigenic mass ELISA and adjusted to the same concentrations of antigens in the commercially available vaccine Porcilis App™ orresponding to approximately 50 μg of ApxI and 25 μg of ApxII per dose

(2 ml) of vaccine. (iv) Vaccine IV (Porcilis App™) was a positive protection control, using the commercially available subunit vaccine Porcilis App™ (Intervet International).

2.4. Vaccinations and challenge

Vaccination was done intramuscularly. For each vaccine, three 6 weeks old SPF landrace pigs were immunized on day 0 (zero) and subsequently received a booster vaccination on day 28. Three control animals (group V) received injections of adjutants on the same days. Two weeks after booster vaccination, on day 42, control sera were taken and then the pigs were challenged with *A. pleuropneumoniae* serotype 1 strain 1-L-452. The bacteria for challenge were grown for 6 h and washed twice by centrifugation at 10,000 × *g* for 15 min and re-suspension in the original volume of PBS buffer. Pigs of all groups were exposed at the same time for 15 min to the bacteria in an aerosol using a De Vilbiss nebulizer [23]. After challenge, fever and respiratory symptoms were recorded. Two weeks later, on day 56, the pigs were slaughtered, unless they died of the infection or had to be euthanized prior to this date. Blood was taken on the day of immunization, on day 42 (prior to challenge) and from the surviving animals at day 56. Dead and euthanized animals were subjected to post-mortem examination for typical lung lesions and recovery of challenge bacteria from lungs and tonsils.

2.5. Production of recombinant PalA'His

In order to specifically monitor the antigenic response to PalA in vaccinated pigs, we have produced an antigenically specific recombinant peptide consisting of the hydrophilic central- and C-terminal part of PalA containing the 106 most C-terminal amino acids (11.54 kDa) fused to 6 N-terminal and 10 C-terminal histidine residues. The corresponding part of the *palA* gene was amplified using the oligonucleotide primers X89009-L (cgc*catatg*-CAAACTCGTTACACCACT) and X89009-R (cgc*ggatcc*-GTATTCTAATACTGCACG). The primers were designed to contain recognition sites for the restriction enzymes *Nde*I and *Bam*HI (shown in italic letters) by the addition of supplementary nucleotides (shown in lower case). This procedure allowed the PCR amplification product to be cloned into the *Nde*I and *Bam*HI cloning sites of vector pETHIS-1. PCR was carried out with a DNA thermal cycler (GeneAmp 9600; Perkin-Elmer Cetus, Norwalk, CT, USA) in a 50 μl reaction mixture containing 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 170 μM of each dNTP, 0.25 μM forward and reverse primers, 0.5 units *Pwo* polymerase and 5 ng genomic DNA of *A. pleuropneumoniae* strain 4074. The amplification was carried out for 35 cycles (30 s denaturation at 94 °C, 30 s annealing at 50 °C, 1 min elongation at 72 °C). The PCR product was purified using the QIA quick PCR purification kit (Qiagen, Basel, Switzerland), cut with the restriction enzymes *Nde*I and *Bam*HI and ligated to *Nde*I and *Bam*HI digested pETHIS-1. Ligated DNA was transformed into *E. coli* strain XL1-blue MRF and positive clones were selected by colony hybridization using a digoxigenin labelled DNA probe for *palA* [11]. A positive clone, named pJFF-PalA1H, was sequenced to verify the integrity of the cloned segment of *palA* and the fusions with the poly-His codons of pETHIS-1. For biosynthesis of recombinant PalA'His peptide, plasmid pJFFPalA1H was introduced into *E. coli* BL21 (DE3) for expression, which was induced by addition of 1 mM IPTG at mid-exponential phase and incubation for a further 2.5 h. Following induction, the poly-histidine tailed fusion protein PalA'His was purified from cell extracts dissolved with 6 M guanidine hydrochloride using Ni^{2+} chelate affinity chromatography (Qiagen) according to the manufacturer's instructions. The bound PalA'His was eluted by slowly decreasing the pH from 8.0 to 4.5 with 50 mM potassium phosphate buffer, 300 mM NaCl, 6 M guanidine hydrochloride. Following elution at pH 4.5, the fusion protein was dialyzed against 50 mM phosphate buffer, 300 mM NaCl, pH 7.5. The fractions were analyzed by standard SDS-PAGE [18] and protein concentrations were measured by the method of Bradford [20].

2.6. Serological assays

In order to monitor the immune response to PalA in vaccinated pigs, we have developed an immunoblot assay using purified recombinant PalA'His as an antigen. Samples of 100 μg PalA'His were separated on 14% SDS-PAGE of 8 cm × 8 cm and subsequently blotted onto nitrocellulose membranes (BioRad, Hercules, CA, USA, product no. 162-0112). The dried membranes were cut into 20 strips with a width of 4 mm in order to get immunoblot strips each containing 5 μg of PalA'His. The strips were reacted with pig sera diluted 1:500, using the standard immunoblot procedure [18]. Phosphatase labelled goat antibodies, directed against pig IgG (Kirkegaard & Perry, Gaithersburg, MD, USA; product no. 051401), diluted 1:2000, followed by addition of nitroblue tetrazolium and bromochlorindolyl phosphate in alkaline phosphate buffer [18], were used to visualize bound antibodies. Each lot of immunoblot strips was controlled using rabbit anti-PalA antibodies [11] at a dilution of 1:1000 and phosphatase labelled goat antibodies directed against rabbit IgG (Kirkegaard & Perry, product n . 075-1506) diluted 1:2000.

Specific antibody titers in serum against ApxI, ApxII, ApxIII and 42 kDa OMP were determined by indirect ELISA as described [24]. This ELISA is based on the antigens ApxI, ApxII, ApxIII and 42 kDa OMP that were purified from *A. pleuropneumoniae* strains with serotypes not related to the production strains used for vaccine antigen production, in order to avoid possible cross-reaction with c ntaminating polysaccharides. Concentrations of antigen preparations were determined in antigenic mass ELISA (toxins) or SDS-PAGE (OMP), relative to reference

preparati ns. The antigenic mass ELISA determines the concentration in arbitrary units relative to the reference preparations. The concentrations of the proteins used for coating of the micro-titer plates for antibody ELISA were in the order of 1 μg/ml. Final adjustments were made depending on antibody titers obtained with positive and negative reference sera. Routinely samples were measured twice and the mean values were reported. The differences of the two measurements were below 10%.

3. Results

3.1. Immune response to PalA in vaccinated pigs

The results of the analysis of anti-PalA antibodies in the blood sera by immunoblots with PalA'His are shown in Fig. 1. All pigs were free of anti-PalA antibodies before vaccination. Pigs vaccinated with vaccines I or II, which contained PalA alone or in combination with ApxI + ApxII, showed a weak reaction 28 days after the first vaccination, and a strong anti-PalA reaction 2 weeks after the booster vaccination. One pig belonging to group II (#176), from which a blood sample was taken before slaughter, showed an anti-PalA reaction on day 56 (Fig. 1). Groups III and IV of pigs that were vaccinated with vaccine containing ApxI + ApxII or with the commercial vaccine respectively, did not show anti-PalA antibodies after first vaccination (day 28) or after booster vaccination (day 42). One pig (#181) of group III showed a very weak anti-PalA reaction 2 weeks after challenge on day 56, which is thought to be due to the challenge with *A. pleuropneumoniae* (Fig. 1).

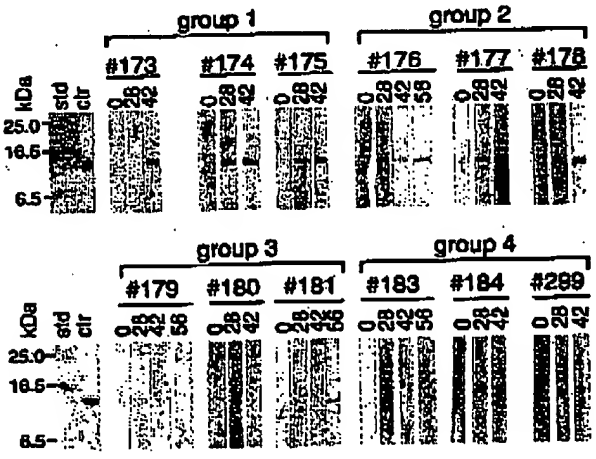

Fig. 1. Serological analysis of anti-PalA antibodies. Immunoblot strips each containing 5 μg PalA'His protein incubated with the different sera before and after vaccination are shown. Figures in the horizontal position indicate the pig numbers, figures in the vertical position indicate days after vaccination. Group I included pigs #173, #174 and #175 vaccinated with vaccine I (PalA); group II included pigs #176, #177 and #178 vaccinated with vaccine II (PalA + ApxI + ApxII); group III included pigs #179, #180 and #181 vaccinated with vaccine III (ApxI + ApxII); group IV included pigs #183, #184 and #299 vaccinated with vaccine IV (Porcilis App™, (Intervet) containing ApxI, ApxII, ApxIII and 42 kDa Omp). Note that pigs #173, #174, #175 and #177 died or were euthanised before the end of the experiment. Std: broad range pre-stained protein markers (New England Biolabs, Beverly, Mass, USA; no. 77085); the position of the molecular masses of 25, 16.5 and 6.5 kDa are indicated.

Table 1
Antibody responses after two vaccinations, measured 2 weeks after booster vaccination and before challenge

Vaccine (content)	Antibody titer against antigen			
	ApxI	ApxII	ApxIII	42 kDa OMP
I (PalA)	<2[a]	<2[a]	<2.0[a]	<2.0[a]
II (PalA + ApxI + ApxII)	3.3	2.6	<2.0[a]	<2.0[a]
III (ApxI + ApxII)	3.5	2.6	<2.0[a]	<2.0[a]
IV (Porcilis App™)	3.2	2.8	2.8	2.4
V (Control)	<2.0[a]	<2.0[a]	<2.0[a]	<2.0[a]

ELISA titers are expressed as the logarithm ($\log_{10}$) of the reciprocal of the highest dilution of serum with an OD above that of the preimmune serum for each pig diluted 1:100 as defined by [24]. Figures represent the mean values of two measurements.

[a] Indicates below detection level.

The antibody responses to vaccination against ApxI, ApxII, ApxIII and 42 kDa OMP were as expected for the various vaccines containing these antigens, as shown in Table 1. The comparison of antibody titers against ApxI and ApxII after vaccination with PalA (group II) or without PalA (group III) added, showed that the serological titers of ApxI and ApxII were not affected by the presence of PalA in the vaccine.

3.2. Protection against infection with A. pleuropneumoniae

The susceptibility of the pigs to *A. pleuropneumoniae* was assessed by challenge of a non-vaccinated group of pigs. In this group, all pigs showed high fever after the challenge as well as abdominal respiration and coughing, which are typical signs for pleuropneumonia (Table 2). One of the three pigs died 6 days post-infection. Upon necropsy, all pigs showed lung lesions affecting, on average, 50–75% of the lungs. These results showed that the challenge with *A. pleuropneumoniae* serotype 1 strain resulted in typical signs of porcine pleuropneumonia under the given experimental conditions.

The group of pigs vaccinated with the commercial vaccine Porcilis App™ (vaccine IV) showed significantly fewer clinical signs after challenge with low or no fever and virtually no respiratory distress. No mortality occurred in this group and no or only minor lung lesions affecting less than 25% of the lung were detected. These results testify to the high protection obtained with the subunit vaccine.

In the group of pigs vaccinated with ApxI and ApxII alone (vaccine III), a high level of protection against infection with *A. pleuropneumoniae* serotype 1 was observed. This was similar to the group vaccinated with the commercial subunit

Table 2
Effect of challenge with *A. pleuropneumoniae* serotype 1 on pigs vaccinated with various vaccines

Vaccine (content)	Pig no.	Fever[a]	Respiratory distress[b]	Mortality (day p.i.)[c]	Lung lesion score[d]	Re-isolation[e]	
						Lung	Tonsil
I (PalA)	173	nd	3	+ (1)	4	+	+
	174	nd	3	+ (1)	4	+	–
	175	2	3	+ (2)	4	+	Cont.
	avg	2	3	3/3 (1.3)	4	3/3	1/2
II (PalA + ApxI + ApxII)	176	2	2	–	2	+	+
	177	0	3	+ (2)	4	Cont.	Cont.
	178	2	2	–	3	+	+
	avg	1.3	2.3	1/3 (2)	3	2/2	2/2
III (ApxI + ApxII)	179	0	0	–	0	–	+
	180	0	0	–	0	–	+
	181	1	1	–	0.5	+	–
	avg	0.3	0.3	0/3	0.2	1/3	2/3
IV (Porcilis App™)	299	1	1	–	1	+	–
	183	0	0	–	0	–	+
	184	1	0	–	1	+	–
	avg	0.7	0.3	0/3	0.6	2/3	1/3
V (Control)	185	2	2	+ (6)	4	+	+
	186	2	2	–	2	–	+
	189	2	2	–	3	+	+
	avg	2	2	1/3 (6)	3	2/3	3/3

[a] Scale used: (0) no fever (<40 °C); (1) fever (>40–41 °C); (2) high fever (>41 °C); (nd) could not be determined due to the rapid death of the animal.
[b] Scale used: (0) no distress, normal respiration; (1) increased respiration rate; (2) abdominal respiration and/or coughing; (3) dyspnea.
[c] (+) Indicates dead pig or pig euthanised because of severe clinical signs (number in brackets gives the day of death post-infection); (–) indicates that the pig did not die.
[d] Scale used: (0) no lesions; (1) 1–25% of the lungs affected; (2) 26–50% affected; (3) 51–75% affected; (4) 76–100% affected (severe lesions).
[e] (+) Indicates animal from which the challenge strain was re-isolated; Cont.: isolation of *A. pleuropneumoniae* not possible since culture was contaminated by other bacteria; (–) indicates that no *A. pleuropneumoniae* could be isolated.

vaccine. This group showed no mortality, only one pig with mild fever and increased respiration rate and virtually no lung lesions after necropsy.

Vaccine II, containing PalA added to ApxI and ApxII, showed no protection. On average, the pigs showed clinical signs similar to the non-vaccinated control group. In this group, the mortality rate was similar to that of the control group. In addition, lung lesions in this group showed the same scores as in the non-vaccinated group.

Vaccination of pigs with purified PalA alone (vaccine I) showed no protection, but rather severe symptoms and rapid death after challenge with *A. pleuropneumoniae* serotype 1. All three pigs died within 1 or 2 days after challenge and subsequent necropsy revealed severe lung lesions. In comparison with the non-vaccinated control group, vaccination of pigs with PalA alone resulted in a significant increase in mortality, three out of three PalA vaccinated animals died, compared to one animal out of three that died from the control group ($\chi^2 = 3.0$; $P = 0.08$) (Table 2). Most significant, was the sudden death after challenge of the PalA vaccinated pigs one to 2 days post-infection, while in the control group one pig died 6 days post-infecti n (Table 2). Upon necropsy, all pigs vaccinated with PalA showed very severe lung lesions (score 4 with 75–100% of the lungs affected) compared to one animal out of three in the control group (Table 2).

From pigs of all groups, the challenge strain could be re-isolated after necropsy from lungs and from tonsils, showing no differences between the control group and the different vaccinated groups. From a few animals, re-isolation of the challenge strain was hindered due to strong growth of other bacteria on the culture media (Table 2).

4. Discussion

The family of PAL proteins has been characterized as proteins constituting integral parts of the outer membrane of many Gram-negative bacteria. They are highly conserved within given bacterial species. Moreover, they show strong similarities between different bacterial species. They are described as very strongly antigenic proteins in several pathogenic bacteria such as *A. pleuropneumoniae* [11], *H. influenzae* [25,26], *Legionella pneumophila* [27], *P. multocida* [15], *Campylobacter jejuni* [28] and *Brucella abortus* [29]. The prototype of PAL, the peptidoglycan associated lipoprotein PAL of *E. coli* was shown to form complexes,

ne type together with the outer membrane proteins TolA, TolQ and TolR, and a second type with the periplasmic protein TolB, in order to maintain the outer membrane integrity by anchoring the outer membrane to the peptidoglycan layer [30]. Mutants deficient in the PAL protein appear to be debilitated for growth under certain conditions. A PAL-deficient mutant of *Haemophilus ducrei* was shown to display a reduced capacity for pustule formation compared to its wild type parent when injected in human volunteers [31]. In addition, the mutant was more susceptible to the antibiotics Erythromycin, Cefotaxime and Ciprofloxacin than the wild type strain and could not be reisolated from pustules in contrast to the wild type strain [31]. The PAL proteins seemed therefore to be valuable targets for immune protection. Several reports of indirect evidence indicate that protein P6 (alternatively named Hi-PAL) of *H. influenzae* is involved in the induction of protective immunity against *H. influenzae* infections. They include studies showing that antibodies to P6 are protective in the infant rat model against invasive *H. influenzae* type b [32]; the demonstration of bactericidal activity for *H. influenzae* of an antibody to P6 immunopurified from human serum [33]; and the fact that rabbit antiserum raised to purified P6 is bactericidal for a broad range of *H. influenzae* strains including many non-typable *H. influenzae* [16]. From these results it was thought that P6 might be a valuable subunit in vaccines against *H. influenzae* infections. Moreover, PAL of other pathogens were considered as appropriate antigens in vaccines. However, no protection against avian cholera was obtained by vaccination with recombinant P6-like protein from *P. multocida* [34].

In our approach to examine the effect of a PAL protein as candidate for a vaccine, we have taken advantage of a well established challenge model of pigs with *A. pleuropneumoniae* to test the efficacy of PalA alone or PalA in combination with known protective antigens as vaccines against porcine pleuropneumonia. In our study, a small number of animals were tested for ethical reasons and, therefore, it does not allow for thorough statistical analysis. However, our data clearly show that pigs, which developed antibody titers against PalA after immunization, showed more significant symptoms, a much higher mortality and died much faster after challenge with *A. pleuropneumoniae* than unvaccinated control pigs. The more severe lung lesions found after necropsy in the PalA vaccinated group further highlighted this observation. Hence the higher mortality and the faster occurring death in the PalA vaccinated group seemed to be an aggravation of the pleuropneumonia and was not due to secondary effects like septic shock. The difference in protective efficacy between the vaccine containing ApxI and ApxII and the vaccine with ApxI, ApxII and PalA is of particular interest. The pigs vaccinated with the two cytotoxins ApxI and ApxII were well protected against challenge with *A. pleuropneumoniae* serotype 1, like the group that was vaccinated with the commercially available subunit vaccine Porcilis App™. In contrast, the protective efficacy of ApxI and ApxII vaccine was completely lost when it was supplemented with PalA, as shown in the group of pigs vaccinated with vaccine II. H nce, PalA antibodies significantly reduce the protective effect of anti-ApxI and anti-ApxII antibodies. The mechanism behind this negative effect of PalA on protective immunity is not known. However, we rule out the possibility that PalA would have had a negative effect on the induction of antibodies against ApxI and ApxII, as the anti ApxI and ApxII titers are the same in the presence or absence of PalA. Since PalA is well conserved in all serotypes of *A. pleuropneumoniae*, the effect is expected to occur with any of the serotypes.

Although the limited number of animals used did not allow us to perform dose-dependence studies, we conclude that PalA should be absent in vaccines against *A. pleuropneumoniae*. Our study does not permit us to extrapolate whether other PAL antigens such as P6 of *H. influenzae* would yield similar effects. However, PalA shows very high similarity to P6 of *H. influenzae* (73% identical and 82% similar aa) and to P6-like protein of *P. multocida* (72% identical and 97% similar aa). Vaccination with other PAL proteins could therefore result in similar negative effects, or as in the case of the P6-like protein of *P. multocida*, give no protection [34]. When using whole cell preparations of bacterial cultures (bacterin vaccines), it must be noted that the concentration of PAL proteins varies depending on the mode of cultivation and preparation of the bacteria, and might therefore vary from one batch to another. Consequently, this could be an explanation of the variations in protective efficacy of certain bacterin vaccines, which are currently observed. Since the negative effect of PAL proteins in vaccines seems to be non-predictable, it would be advisable to avoid PAL proteins in vaccines unless specific evidence for a positive effect on protection is found. The existence of such negatively acting components gives further support to the need for development of well defined subunit vaccines against bacterial infections.

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References

[1] Taylor DJ. *Actinobacillus pleuropneumoniae*. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, editors. Diseases of swine. Ames, Iowa USA: Iowa State University Press; 1999. p. 343–54.

[2] Blackall PJ, Klaasen HBLM, van den Bosch H, Kuhnert P, Frey J. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet Microbiol 2002;84:47–52.

[3] Frey J. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends Microbiol 1995;3(7):257–61.

[4] Schaller A, Kuhn R, Kuhnert P, Nicolet J, Anderson TJ, MacInnes JI, et al. Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology 1999;145:2105–16.

[5] Schaller A, Djordjevic SP, Eamens GJ, Forbes WA, Kuhn R, Kuhnert P, et al. Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene *apxIVA*. Vet Microbiol 2001;79(1):47–62.

[6] Kobisch M, van den Bosch JF. Efficacy of an *Actinobacillus pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society Congress, The Hague, The Netherlands, vol. 12; 1992. p. 216.

[7] van den Bosch JF, Pennings AMMA, Cuijpers MECM, Pubben ANB, van Vugt FGA, van der Linden MFI. Heterologous protection induced by an *A. pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society Congress, Lausanne, Switzerland, vol. 11; 1990. p. 11.

[8] Martelli P, Guadagnini PF, Foccoli E, Ballarini G. Efficacy of an *Actinobacillus pleuropneumoniae* subunit vaccine in the control of pleuropneumonia: a field trial. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Bologna, Italy; 1996. p. 214.

[9] Pommier P, Ridremont B, Wessel-Roberts S, Keïta A. Field study into efficacy of a new *Actinobacillus pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Bologna, Italy; 1996. p. 206.

[10] Valks MMH, Nell T, van den Bosch JF. A clinical field trial in finishing pigs to evaluate the efficacy of a new APP subunit vaccine. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Italy; 1996. p. 208.

[11] Frey J, Kuhnert P, Villiger L, Nicolet J. Cloning and characterization of an *Actinobacillus pleuropneumoniae* outer membrane protein belonging to the family of PAL lipoproteins. Res Microbiol 1996;147(5):351–61.

[12] Rapp VJ, Ross RF. Antibody response of swine to outer membrane components of *Haemophilus pleuropneumoniae* during infection. Infect Immun 1986;54:751–60.

[13] Deich RA, Metcalf BJ, Finn CW, Farley JE, Green BA. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J Bacteriol 1988;170:489–98.

[14] Nelson MB, Apicella MA, Murphy TF, Vankeulen H, Spotila LD, Rekosh D. Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. Infect Immun 1988;56:128–34.

[15] Kasten RW, Hansen LM, Hinojoza J, Bieber D, Ruehl WW, Hirsh DC. *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *Haemophilus influenzae*. Infect Immun 1995;63:989–93.

[16] Green BA, Metcalf BJ, Quinn Dey T, Kirkley DH, Quataert SA, Deich RA. A recombinant non-furry acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both nontypeable and type b *H. influenzae*. Infect Immun 1990;58:3272–8.

[17] Munson Jr RS. *Haemophilus influenzae*: surface antigens and aspects of virulence. Can J Vet Res 1990;54(Suppl):S63–7.

[18] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in molecular biology. New York, NY: Wiley; 1999.

[19] Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 1989;8:151–6.

[20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[21] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[22] Frey J, Nicolet J. Regulation of hemolysin expression in *Actinobacillus pleuropneumoniae* serotype 1 by Ca^{2+}. Infect Immun 1988;56:2570–5.

[23] Sebunya TN, Saunders JR, Osborne AD. A model aerosol exposure system for induction of porcine *Haemophilus pleuropneumania*. Can J Comp Med 1983;47:48–53.

[24] Nielson R, van den Bosch JF, Plambeck T, Sorensen V, Nielsen JP. Evaluation of an indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to the Apx toxins of *Actinobacillus pleuropneumoniae*. Vet Microbiol 2000;71(1–2):81–7.

[25] Yamanaka N, Faden H. Antibody response to outer membrane protein of nontypeable *Haemophilus influenzae* in otitis-prone children. J Pediatr 1993;122:212–8.

[26] Yamanaka N, Faden H. Local antibody response to P6 of nontypeable *Haemophilus influenzae* in otitis-prone and normal children. Acta Oto-Laryngol 1993;113:524–9.

[27] Engleberg NC, Howe DC, Rogers JE, Arroyo J, Eisenstein BI. Characterization of a *Legionella pneumophila* gene encoding a lipoprotein antigen. Mol Microbiol 1991;5:2021–9.

[28] Burnens A, Stucki U, Nicolet J, Frey J. Identification and characterization of an immunogenic outer membrane protein of *Campylobacter jejuni*. J Clin Microbiol 1995;33:2826–32.

[29] Tibor A, Weynants V, Denoel P, Lichtfouse B, De Bolle X, Saman E, et al. Molecular cloning, nucleotide sequence, and occurrence of a 16.5-kilodalton outer membrane protein of *Brucella abortus* with similarity to pal lipoproteins. Infect Immun 1994;62:3633–9.

[30] Bouveret E, Benedetti H, Rigal A, Loret E, Lazdunski C. In vitro characterization of peptidoglycan-associated lipoprotein (PAL)-peptidoglycan and PAL-TolB interactions. J Bacteriol 1999;181(20):6306–11.

[31] Forney KR, Young RS, Bauer ME, Katz BP, Hood AF, Munson Jr RS, et al. Expression of peptidoglycan-associated lipoprotein is required for virulence in the human model of *Haemophilus ducreyi* infection. Infect Immun 2000;68(11):6441–8.

[32] Munson Jr RS, Granoff DM. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect Immun 1985;49(3):544–9.

[33] Murphy TF, Bartos LC, Rice PA, Nelson MB, Dudas KC, Apicella MA. Identification of a 16,600-dalton outer membrane protein on nontypeable *Haemophilus influenzae* as a target for human serum bactericidal antibody. J Clin Invest 1986;78(4):1020–7.

[34] Kasten RW, Wakenell PS, Ahmad S, Yilma TD, Hirsh DC. Lack of protection against avian cholera by vaccination with recombinant P6-like protein from *Pasteurella multocida*. Avian Dis 1997;41(4):972–6.

PATENT Docket No.: 1038-1160

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Loosmore, et al.
Application No.:		09/857,843
Filing Date	:	September 17, 2001
Title	:	Multi-Component Vaccine Comprising At Least Two Antigens From Haemophilus influenzae To Protect Against Disease
Grp./AU	:	1645
Examiner	:	Jana A. Hines

November 26, 2003

APPEAL BRIEF AND REQUEST FOR EXTENSION OF TIME
TOTAL PAGES 75

BY FACSIMILE 703-872-9307

The Commissioner of Patents and Trademarks
BOX AF,
Washington, D.C.
20231, U.S.A.

Dear Sir:

Introduction

This Appeal Brief is submitted pursuant to applicant's appeal from a Final Rejection of claims 1-5, 25 and 27 dated December 31, 2002. A Notice of Appeal was filed on June 3, 2003. The enclosed Credit Card Payment Form includes the prescribed fee. In the event of underpayment or overpayment please apply any additional charges or refunds to USPTO Deposit Account Number 500715. Three copies of the Appeal Brief are provided herewith.

Extension of Time

Petition is hereby made under the provisions of 37 CFR 1.136(a) for an extension of four months of the period for filing this Appeal Brief. The enclosed Credit Card Payment

Form includes the prescribed fee. In the event of underpayment or overpayment please apply any additional charges or refunds to USPTO Deposit Account Number 500715.

(1) **Real Party of Interest**

The real party of interest with respect to this patent application is Aventis Pasteur Limited. Assignments from the inventors to Aventis Pasteur Limited are recorded at Reel 013317/0711, 0724 and 0733 on September 20, 2002.

(2) **Related Appeals and Interferences**

The appellants, the appellants' legal representatives and assignee, are unaware of any pending appeals or interferences which will directly affect or be affected by or have a bearing on the Board's decision in the pending appeal.

(3) **Status of Claims**

This application was filed with claims 1-26. In the response dated September 25, 2002 to the Office Action of March 27, 2002 claims 6-24 and 26 were cancelled, claim 25 amended, and new claim 27 added.

Claims 1-5, 25 and 27 were finally rejected in an Office Action dated December 31, 2002. Claims 1-5, 25 and 27 are pending and the subject of this appeal and appear in Appendix I hereto.

(4) **Status of Amendments**

This application was filed with claims 1-26. Claims 1-5, 25 and 27 are pending and no amendments were filed subsequent to this final rejection.

(5) **Summary of Invention**

The present invention is directed to an immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae*, including otitis media. The composition comprises at least two different antigens of *Haemophilus influenzae*, at least one of which antigens is an adhesin (claim 1) and wherein said adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae*, (claim 2),

particularly an HMW 1 or HMW 2 protein of the non-typeable strain (claim 3), and said antigen which is not an adhesin is a non-proteolytic heat shock protein of a strain of Haemophilus influenzae (claim 4) and wherein the heat shock protein is an analog of *Haemophilus influenzae* Hin47 protein having a protease activity which is less than about 10% of that of the natural Hin47 protein (claim 5). The invention is further directed to compositions where the HMW protein is recombinantly produced and said antigen which is not an adhesin is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of the natural Hin47 protein in which the Histidine at amino acid position 91 is replaced by Alanine (claim 27). The present invention is further directed to a method of immunizing a host against disease caused by infection with *H. influenzae* (claim 25).

(6) **Issues**

The issues for consideration is the rejection of, claims 1-5 and 25 under 35 U.S.C. 112 1st paragraph and claims 1-5, 25 and 27 under U.S.C. 103(a) as being unpatentable over Barenkamp et al in view of Loosmore et al.

(7) **Grouping of Claims**

All claims do not stand or fall together, but rather each claim is individually patentable.

(8) **Argument**

(a) Background to the Invention

Haemophilus influenzae is the cause of several serious human diseases, such as meningitis, epiglottitis, septicemia and otitis media. There are six serotypes of *H. influenzae,* designated a to f, that are identified by their capsular polysaccharide. *H. influenzae* type b (Hib) was a major cause of bacterial meningitis until the introduction of several Hib conjugate vaccines in the 1980's. Vaccines based upon *H. influenzae* type b capsular polysaccharide conjugated to diphtheria toxoid, tetanus toxoid, or *Neisseria meningitidis* outer membrane protein have been effective in reducing *H. influenzae* type b-induced meningitis. The other serotypes of *H. influenzae* are associated with invasive disease at low frequencies, although there appears to be an increase in the incidence of disease caused by these strains as the incidence of Hib disease

declines. Non-encapsulated or non-typeable *H. influenzae* (NTHi) are also responsible for a wide range of human diseases including otitis media, epiglottitis, pneumonia and tracheobronchitis. The incidence of NTHi induced disease has not been affected by the introduction of the Hib vaccines.

Otitis media is the most common illness of early childhood, with 60 to 70% of all children, of less than 2 years of age, experiencing between one and three ear infections. Chronic otitis media is responsible for hearing, speech and cognitive impairments in children. *H. influenzae* infections account for about 30% of the cases of acute otitis media and about 60% of chronic otitis media. In the United States alone, treatment of otitis media costs between 1 and 2 billion dollars per year for antibiotics and surgical procedures, such as tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. It is estimated that an additional $30 billion is spent per annum on adjunct therapies, such as speech therapy and special education classes. Furthermore, many of the causative organisms of otitis media are becoming resistant to antibiotic treatment. An effective prophylactic vaccine against otitis media is thus desirable.

(b) The Present Invention

Having regard to the above Background, it would be desirable to provide efficacious combination vaccines comprising *H. influenzae* components containing selected relative amounts of selected antigens. The present invention provides an immunogenic composition for conferring protection in a host against disease caused by infection with *H. influenzae,* including otitis media.

The immunogenic composition comprises at least two different antigens of *H. influenzae,* one of which is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae* at least one of which is an adhesin as claimed in claim 1, and all claims dependant thereon.

Claim 2 recites that the adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *H. influenzae*. Claim 3 recites that the HMW protein is a HMW 1 or HMW 2 protein of a non-typeable strain of *H. influenzae*. Claim 4 recites that the antigen which is not an adhesin is a non-proteolytic heat shock protein of a non-typeable strain of *H. influenzae*. Claim 5 recites that the heat shock protein is an analog of *Haemophilus influenzae* Hin47 protein having a protease activity which is less than about 10% of that of the natural Hin47 protein.

Claim 27 further recites that the Hin47 protein having a decreased protease activity which is less than about 10% of that of the natural Hin47 protein in which the Histidine at amino acid position 91 is replaced by Alanine. The applicants data supports such results.

(c) Rejection of claims 1-5 and 25 under 35 U.S.C. 112 1st paragraph.

The HMW proteins are described in Barenkamp et al cited by the Examiner in the prior art rejection while the Hin47 analogs are described in the Loosmore et al reference cited by the Examiner in the prior art rejection. In the Barenkamp et al reference, there is described both the isolation of the HMW protein from natural-source materials and recombinant production. It is submitted, that the enablement is not limited to recombinantly-produced HMW protein.

In the Loosmore et al reference, there is described the manner of producing the non-proteolytic analog of Hin47 protein. In this respect, at least one amino acid contributing to protease activity is deleted or replaced by a different amino acid. The Loosmore et al reference describes how to identify such amino acid by comparison to known proteases. The reference specifically describes that the deleted or replaced amino acid may be selected from amino acids 195 to 201 and specifically describes replacement of Serine-197 with alanine, other specific amino acid mutations described are Histidine-91 replaced with alanine, and lysine or arginine-121 replaced with alanine. The immunogenic properties of these various mutants are described in Loosmore et al. Based on this information, there is no reason to suppose that any other non-proteolytic analog would not also function in the same manner as the specific H9IA Hin47 analog utilized in the experiments described in the application (page 18 lines 4-12). It is submitted that enablement is not limited to the specific H91A Hin47 analog, but rather extends at least to any non-proteolytic analog of the Hin47 protein. The Examiner indicates that the objection of lack of enablement is based, to some extent, upon lack of guidance as to how to determine compositions other than that specifically identified by the Examiner. It is submitted that such is not the case.

Specifically, the specification tells a person skilled in the art that two different antigens of *Haemophilus influenzae* are employed and that one of them has to be an adhesin and the other does not. Testing to determine if an antigen is an adhesin or not an adhesin is within the skill of the art. In this regard, the Examiner's attention is directed to the experimentation described in Barenkamp.

In addition, the person skilled in the art is advised that one such adhesin protein is the HMW protein, where that is described and how to produce it both from natural-source materials and recombinantly (see page 2, line 23 to page 3, line 23). In addition, the person skilled in the art is advised that one such nonadhesin protein is a non-proteolytic analog of Hin47 protein or other non-proteolytic heat shock protein and how to produce such an analog (see page 3, line 14 to page 3 line 31).

Furthermore applicants have given guidance to one skilled in the art to test if a composition is an immunogenic composition (see example 4 pages 18 to 20 of the present application).

Having regard to the foregoing discussion, it is submitted that claims 1 to 5 and 25 are fully enabled by the disclosure.

(d) Rejection of claims 6-24 under 35 USC 103(a).

Claims 1 to 5, 25 and 27 have been finally rejected under 35 USC 103(a) as being unpatentable over Barenkamp (WO 97/36914) in view of Loosmore et al (US Patent 5,506,139).

Claim 1 defines an immunogenic composition for conferring protection in a host against disease caused by *Haemophllus influenzae* comprising at least two different antigens of *Haemophilus influenzae*.

- one of which antigens is an adhesin
- the other of which antigens is not an adhesin.

The Examiner has identified Barenkamp et al as describing a *Haemophilus influenzae* protein which is an adhesin and Loosmore as describing a *Haemophllus influenzae* protein which is not an adhesin. The applicants position is that neither reference provides the motivation to combine the two immunogens in a single composition, as required by claim 1.

Barenkamp teaches high molecular weight proteins of non-typeable *H. influenzae* identified as HMW1, HMW2, HMW3 and HMW4, which are characterized by molecular weight and sequence information. Loosmore et al teach an analog of *H. influenzae* Hin47 protein with reduced protease activity. It is submitted that these references lack any motivation to combine two different antigens of *H. influenzae*, namely a non-proteolytic Hin47 protein of Loosmore et

al with the HMW proteins of Barenkamp et al in an immunogenic composition. It is the applicants position that neither reference provides the motivation to combine the two immunogens in a single composition as required by claim 1, and by dependency all claims on appeal.

While suggesting various combinations, there is no suggestion here to combine different proteins derived from the same pathogen, as in applicants claim 1. Again, the references are silent as to any specific combination contemplated.

The cited prior art lacks the motivation to do so. There are vague, non-specified indications in both references to combine other components with the specific immunogen, but there is no specific indication as to what that other component may comprise, other than an adjuvant or materials from the pathogens and/or materials from various strains of the same pathogen.

As the Examiner has pointed out, on page 49, lines 15 to 19 of Barenkamp, it is stated:

> "…. the data suggests the HMW adhesin proteins are potentially important protective antigens which may comprise one component of a multi-component NTHI vaccine."

This passage appears to suggest that only *Haemophilus* proteins which are the HMW adhesin proteins are appropriate components. The non-proteolytic analog of Hin47 is not an adhesin (although initially thought to be adhesin, see col. 2, line 17 of Loosmore et al). (It is pointed out that the Examiner is incorrect in the statement that the adhesin protein "should" comprises one component of the NTHI vaccine. As can be seen from the above quotation, Barenkamp uses the word "may").

Even if the Examiner finds motivation in this passage of Barenkamp to combine the HMW protein with another *Haemophilus* antigen, whether an adhesin or not, such motivation still provides no motivation to select the non-proteolytic Hin47 analog as the other *Haemophilus* antigen.

There have been a significant number of *Haemophilus* proteins identified as vaccine candidates besides the HMW and Hin47 analog proteins. These proteins include the

various outer membrane proteins A to H, lactoferrin and transferrin receptor protein and the P1, P2, P6 and D15 proteins. It is submitted that there is no motivation provided by the cited prior art why a person skilled in the art would specifically select from all the optional possibilities, the non-proteolytic Hin47 analog to specifically combine with the HMW protein.

The Examiner states in the Office Action, quoting In re Kerkhoven, that:

> "The idea of combining them flows logically from their having been individually taught in the prior art."

The "idea of combining them" does not explain why the two materials should be combined when there is selection available. If the two antigens were the only two known antigens of *Haemophilus influenzae*, then there may be some validity to the position taken by the Examiner, but this is clearly not the case here.

In any event, caution is required when considering combining different antigens into immunogenic compositions because of the danger of impairment of the immunogenicity of the individual components one by the other. As may be seen from Applicants data, in Figure 3, immunogenic compositions are provide in which there is no impairment of individual antigenic components.

Furthermore, these results are unexpected in the field of combination vaccines. There is little expectation of success that simply mixing existing vaccine antigens will not result in incompatibilities amongst the various antigens, resulting in loss of stability or reduced potency or indeed a synergistic effect increasing potency. Immune interference cannot be predicted. Others skilled in the art of combination vaccines have found that the preparation of combination vaccines is far from straight forward. For example Cauldfield et al (2001) report on the need for a balanced formulations of vaccine components in the preparation of DTP combination vaccines to circumvent interference with the components. Van den Bosch et al (2003) have also reported that the addition of a potential antigen (Pal A) from *Actinobacillus pleuropneumoniae* can completely eliminate the positive efficacy of known antigens (ApxI and II) when combined (see abstract).

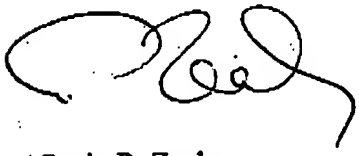
For all these reasons, it is submitted that claims 1 to 5, 25 and 27 are patentable over the applied art and the rejection thereof under 35 USC 103(a) as being unpatentable over

Barenkamp in view of Loosmore et al. cannot be sustained.

Summary

Having regard to the above detailed discussion, it is submitted that the Examiner is in error in rejecting claim 1 to 5, 25 and 27 as being unpatentable under 35 USC 112 1st paragraph and the rejection under 35 USC 103(a) as being unpatentable over the combination of Barenkamp in view of Loosmore et al, should be REVERSED.

Respectfully submitted,



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(9) **APPENDIX I**

CLAIMS APPEALED (09/857,843)

1. An immunogenic composition for conferring protection in a host against disease caused by *Haemophilus influenzae,* comprising:
at least two different antigens of *Haemophilus influenzae,* at least one of which antigens is an adhesin.

2. The immunogenic composition of claim 1 wherein said antigen which is an adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae.*

3. The immunogenic composition of claim 2 wherein said HMW protein is a HMW 1 or HMW2 protein of the non-typeable strain of *Haemophilus influenzae.*

4. The immunogenic composition of claim 1 wherein the antigen of *Haemophilus influenzae* which is not an adhesin is a non-proteolytic heat shock protein of a strain of *Haemophilus influenzae.*

5. The immunogenic composition of claim 4 wherein the non-proteolytic heat shock protein of a strain of *Haemophilus influenzae* is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein.

25. A method of immunizing a host against disease caused by infection with *Haemophilus influenzae,* including otitis media, which comprises administering to the host an immunoeffective amount of a composition as claimed in claim 1.

27. The composition of claim 1 wherein said antigen which is an adhesin is a recombinantly-produced high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus*

influenzae and said antigen which his not an adhesin is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein, in which Histidine-91 is replaced by alanine.

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Immunogenicity of a hexavalent combination vaccine in rhesus monkeys

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Abstract

Preclinical immunogenicity studies were conducted in rhesus monkeys to determine whether there is immune interference in the response to one or more components of a hexavalent vaccine (Hexavac™) that contains antigens from *Haemophilus influenzae* (Hib), hepatitis B (HB), diphtheria (D), tetanus (T), acellular pertussis (aP) and inactivated polio virus (IPV). Antibody responses were measured following co-administration of the components at three separate anatomical sites or administration as a hexavalent combination in a single site. After three injections of the hexavalent vaccine, the peak antibody responses to each component of the vaccine were > 100-fold above pre-immune titers and persisted at levels > 10-fold above pre-immune titers at ≈ 1 year. Immune interference was observed in the peak response to HB, D and pertussis toxin, but was not seen at later time points. The results indicate that the rhesus monkey model may be useful for pre-clinical evaluation of combination vaccines.

Keywords: Combination vaccine; Immune interference; Antigen competition; Non-human primate

1. Introduction

The rationale for the development of combination vaccines has been discussed in recent publications [1–3]. The main benefits are to enhance compliance and vaccine coverage and to reduce overall healthcare costs. An added benefit is to 'make room' in the pediatric vaccination schedule for new vaccines projected for the new millennium [4]. However, experience has shown that the preparation of combination vaccines is far from straightforward. During the development of the DTP combination vaccine, the need for 'balanced' formulations of vaccine components was recognized [5] and careful dose-ranging of the three serotypes of the oral poliovirus vaccine (OPV) was required in order to circumvent interference of the type 2 strain on the immune response to types 1 and 3 [6]. The main impediments to the development of combination vaccines are stability and immunogenicity. Simply mixing existing vaccines can result in incompatibilities among the various antigens, adjuvants, preservatives, stabilizers and excipients, resulting in a loss of stability or reduced potency [7]. A further confounding factor is that of immune interference (also known as antigen competition) which may not always be predicted using animal models. Antigenic competition was first described by Michaelis in 1904 [8], but is still poorly understood.

The objective of the present preclinical immunogenicity studies of Hexavac™ was to determine the antibody response to vaccine component antigens at various times after immunization and to compare the response to the hexavalent vaccine with that induced by administration of Hib, HB and DTaP-IPV at separate anatomic sites. The results indicate that there was a significant difference between experimental and control arms in the peak responses to HB, D and PT. These differences fade with time and there was no significant

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difference in the response to any vaccine component tested at 48–51 weeks post-dose 1. Overall, strong antibody responses were induced to each component of Hexavac™ in rhesus monkeys.

2. Materials and methods

2.1. Experimental animals

The rhesus monkeys (*Macaca mulata*) used in this study were born at the California Regional Primate Center at the University of California at Davis and all immunizations and blood collection procedures were performed at that site. Some monkeys were housed outdoors in social groups, whereas others were maintained indoors, in pairs. Those maintained indoors had a 12:12 h light:dark cycle within a temperature range of ≈17–29°C. Animals were all fed Purina Monkey Chow, 15% protein with fresh produce supplements two to three times per week. Monkeys were identified by tattoos containing unique numbers. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Vaccine composition

The hexavalent vaccine consisted of Hib capsular polysaccharide (polyribosyl ribitol phosphate) conjugated to tetanus toxoid (PRP-T), hepatitis B (HB), surface antigen (HBsAg), diphtheria toxoid (D), tetanus toxoid (T), pertussis filamentous hemagglutinin (FHA), pertussis toxoid (PT) and three serotypes of inactivated poliovirus (IPV) formulated with aluminum adjuvants. Each 0.5 ml dose contained 12 μg PRP-T (expressed in polyoside), 5 μg HBsAg, 30 Lf D, 10 Lf T, 25 μg FHA, 25 μg PT, IPV type 1 (40 D-Ag U), type 2 (8 D-Ag U) and type 3 (32 D-Ag U).

2.3. Vaccination/schedule

One group of rhesus monkeys was immunized with half of the human pediatric dose of the hexavalent combination vaccine (Hexavac™) into a single i.m. site, while a second cohort (control group) of monkeys was injected with half of the human dose of PRP-T (ActHIB®), HBsAg (RECOMBIVAX HB®) and DTacP-IPV at three separate i.m. sites at 0, 4 and 8 weeks, according to the protocol shown in Table 1. The monkeys were weighed at each time point and examined for injection site reactions after each dose of vaccine. In addition, blood samples collected at each time point were monitored for changes in white cell, red cell and platelet levels.

2.4. Serological assays

Sera were collected at week 0, 4, 8, 10 and 48–51 and tested individually for antibody titers against PRP, HBsAg, D, T, FHA and PT. Due to a shortage of sera, serology was not performed to detect antibodies against poliovirus. Anti-PRP (component of Hib) antibody titers were measured using a Farr-type radioimmunoassay (RIA), as previously described [9,10]; responses to HB were determined using a modified Ausab® assay (Abbott Laboratories, N. Chicago, IL), as described elsewhere [11]. Antibodies against FHA [12] and antibody titers against T were measured by ELISA [13]. Diphtheria toxoid antibody titers were assayed by using a neutralization test in comparison to a WHO antitoxin standard [14]. PT antibody titers were also determined by a toxin neutralization test on CHO cell culture [15]. The results are expressed as geometric means. The assays used were originally validated for analysis of human samples and adapted for testing monkey serum without further analytical validation.

2.5. Statistical analysis

At each time point, the estimated GMT ratio for the experimental vaccine group relative to the control group (Hexavac™/Hib + HB + DTaP-IPV) and corresponding two-sample 99% confidence interval for the true GMT ratio are calculated for the response to each antigen, assuming unknown but equal variances between the two groups. If a particular interval excludes

Table 1
Protocol for preclinical immunogenicity testing of Hexavac™ in rhesus monkeys

Group	n	Age at dose 1 (months)	Vaccine	Injection schedule (weeks)	Bleeding schedule (weeks)
1	8[c]	6–12	Hexavac™[a]	0, 4, 8	0, 4, 8, 10, ≈50
2	8	4.8–10.5	ActHIB®+RECOMBIVAX HB®+DTaP-IPV[b]	0, 4, 8	0, 4, 8, 10, ≈50

[a] Injection volume of 0.25 ml in one intramuscular site.
[b] Injection in three separate intramuscular sites (0.25 ml each).
[c] Serum samples from six of eight monkeys were available at the week ≈50 time point.

the value 1, the corresponding comparison between Hexavac™ and Hib + HB + DTaP-IPV is statistically significant; otherwise, it is not. The reason for using a 99% confidence level instead of the usual 95% level is to control the overall false-positive rate (per antigen), which is defined as the probability that at least one of th confidence intervals will exclude the value 1 by chance alone [16].

3. Results

3.1. Serum antibody response to vaccination

Antibody titers to each component of the vaccine (except IPV) were measured at week 0, 4, 8, 10 and 48–50 using sera from individual animals (Fig. 1). At week 4 (post-dose 1), there were no significant differences between groups in antibody titers to any of the antigens tested. Similarly, at week 8 (4 weeks post-dose 2), there were no significant differences among groups with the exception of the response to HB, which was significantly higher in the control group compared with the monkeys injected with the hexavalent combination. As shown in Table 2, at the 10-week time point (2 weeks post-dose 3), there was a significantly higher response to three of the vaccine components (HB, D and pertussis toxin) in monkeys immunized with separate injections of Hib + HB + DTaP-IPV compared with the response of monkeys immunized with the hexavalent combination vaccine. Importantly, at the final time point (week 48–51), which is 38–41 weeks post-dose 3, there was no significant difference in the response to any component of the vaccines.

3.2. Response rate to vaccination

The percentage of responders to components of the vaccine was determined at each bleed time point. In the absence of established 'seroprotective titers' for rhesus monkeys, the accepted human equivalents were used as shown in the legend to Table 3. For pertussis, there is no proven correlate of protection established for humans, therefore, the percentage of seroconverters was used instead. As shown in Table 3, there was no difference in the response rate to vaccination with Hexavac™ compared with separate site administration of Hib + HB + DTaP-IPV except for the response to HB at the 8-week time point. At that time, only 37% (3/8) monkeys responded to Hexavac™ whereas 100% (8/8) responded to the control. These results are consistent with the analysis of the serological titers that showed a significant diff rence in anti-HBs titers at this tim .

3.3. Adverse event monitoring

Animals were monitored for changes in weight or blood cell counts as well as for injection site reactions. No adverse reactions were noted at the site of injection at any time point and there was no adverse effect of vaccination on the weight or blood cell counts of any animals (data not shown).

4. Discussion/conclusion

The results from the present pre-clinical evaluation of Hexavac™ indicate that there was a vigorous response to each component of the vaccine. Even so, there was evidence for interference in the peak response to HB, D and pertussis toxoid when the responses to Hexavac™ were compared with the control group. Four types of immune interference (antigen competition) have been described: (a) sequential; (b) intramolecular; (c) intravirionic; and (d) intermolecular competition.

- Sequential competition occurs when a second antigen (or vaccine) is given shortly after a first antigen (or vaccine) [17]. This form of interference is especially relevant to vaccine dosing schedules.
- Intramolecular competition results from competition among peptides derived from the same protein for binding to MHC Class I or Class II molecules [18].
- Intravirionic competition results when one protein antigen within a virus interferes with the response to a second protein antigen within the same virus [19]. This form of antigen competition can be circumvented by dissociation of the virus into its component parts prior to immunization.
- Intermolecular competition results when one antigen in a mixture interferes with the immune response to a second antigen in a mixture [17,20]. This form of interference is most relevant to the present investigation; however, the mechanism by which this happens is unknown. One possibility is that one or m re components within the combination vaccine become unstable, perhaps due to excipients carried over into the vaccine with a separate component. However, extensive stability studies have been performed on Hexavac™ and the components that had reduced immunogenicity in the combination vaccine (HB, D and PT) were shown to be stable for several years (data not shown). Thus, the decreased response to certain of the vaccine components of Hexavac™ does not appear to be related to a loss of stability of these components, suggesting that the explanation is immune interference due to intermolecular antigen competition.

Although the rhesus monkey model suggests that the response to Hexav c™ is marked by transient interference in response to the HB, D and PT components,

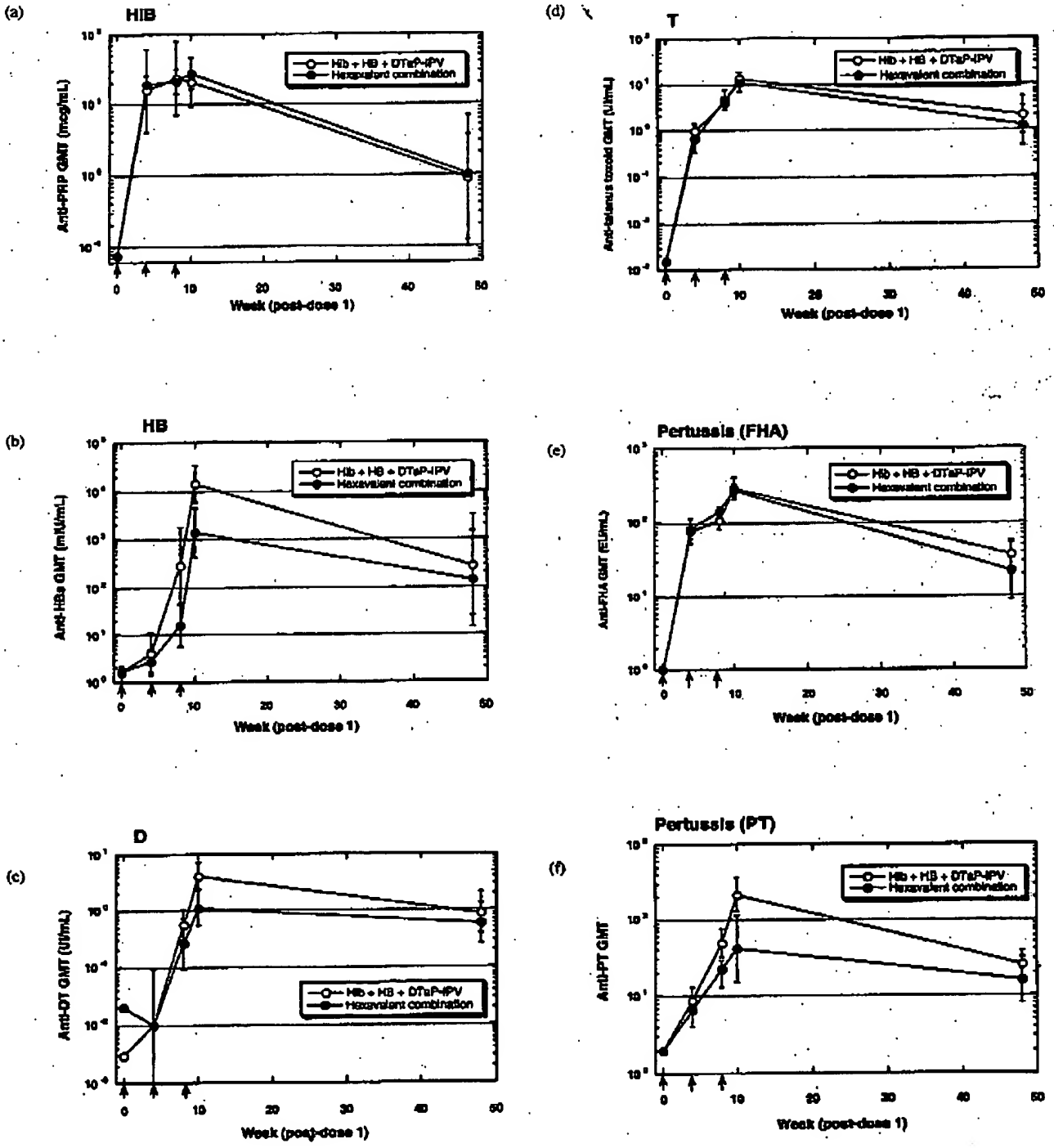

Fig. 1. Antibody response to antigens from Hib, HB, D, T, FHA and PT in rhesus monkeys immunized with Hib + HB + DTaP-IPV at separate sites or with the hexavalent combination vaccine (Hexavac™) at a single i.m. site. Monkeys were immunized at week 0, 4 and 8 and antibody titers were determined on serum collected at week 0, 4, 8 and ≈50. Results are expressed as the geometric mean with 95% confidence intervals.

these apparent differences in potency are unlikely to translate into clinically meaningful diff rences since: (a) the response to each component of the vaccine is > 100-fold higher than the pre-immune titers; (b) the response rate post-dose 3 (percent seroconverters) is equivalent in the two groups; and (c) the difference in titers elicited by Hexavac™ and the control arm became indistinguishable over time. By study week 48–51,

Table 2
Estimated ratios (Hexavac™/Hib+HB+DTaP-IPV) and 99% confidence intervals for the antibody response to Hexavac™ versus control[a]

Antigen	Week (post-dose 1)			
	4	8	10	48–51
HB	0.69 (0.15, 3.21)	**0.06 (0, 0.8)**	**0.1 (0.02, 0.063)**	**0.5 (0.01, 37.61)**
Hib	1.21 (0.2, 7.16)	0.9 (0.23, 3.56)	0.76 (0.23, 2.56)	2.24 (0.14, 36.74)
D	1.49 (0.09, 25.11)	0.47 (0.1, 2.1)	**0.27 (0.08, 0.86)**	0.66 (0.14, 3.04)
T	0.69 (0.25, 1.95)	1.17 (0.55, 2.49)	0.83 (0.39, 1.79)	0.61 (0.11, 3.4)
FHA	1.08 (0.58, 2.03)	1.31 (0.88, 1.95)	0.94 (0.57, 1.57)	0.6 (0.2, 1.82)
PT	0.77 (0.38, 1.55)	0.46 (0.19, 1.09)	**0.19 (0.05, 0.82)**	0.65 (0.26, 1.64)

[a] Any confidence interval excluding the value '1' implies a statistically significant difference. If the upper confidence limit is <1, then the GMT of Hexavac™ is significantly less than the GMT of Hib+HB+DTaP-IPV. Statistically significant results are in bold type.

Table 3
Response rate to Hexavac™ versus the control group[a]

Antigen	Percent responders (Hexavac™, control) at week:				
	Pre	4	8	10	48–51
HB	0, 0	0, 12	37, 100	100, 100	83, 87
Hib	12, 12	100, 100	100, 100	100, 100	83, 75
D	33, 0	17, 0	100, 100	100, 100	100, 100
T	0, 0	0, 0	100, 100	100, 100	100, 100
FHA	0, 0	0, 0	100, 100	100, 100	100, 100
PT	0, 0	62, 87	100, 100	100, 100	100, 100

[a] Criteria for response: HB (>10 mIU/ml); Hib (>0.15 mcg/ml); D (>0.01 IU/ml); T (>0.01 IU/ml); FHA (>4 UE/ml); PT (reciprocal titer >4).

there was no significant difference in the antibody response to any component of the vaccine between monkeys injected with Hexavac™ versus separate site injection of Hib + HB + DTaP-IPV. The kinetics of the response also deserves comment. With the exception of the response to Hib, the titers to each vaccine component increased following each dose of vaccine. The reason for the immediate and vigorous response to Hib is not known, however, the rapid response suggests that the monkeys may have been primed by prior exposure to *Haemophilus influenzae* or to a cross-reacting organism. Subsequent injections of Hexavac™ did not significantly increase the high titers observed after the first injection, which were >10 μg/ml. In previous studies [10], 18–22-month-old rhesus monkeys were found to respond earlier and with higher titers to a Hib vaccine (PedvaxHIB®) than 2–3-month-old monkeys. This suggests that the use of younger monkeys may enable better discrimination among Hib-containing vaccines.

The response rate of rhesus monkeys that received three doses of the vaccine was 100% (at week 10). This compares favorably with the response rates seen in a clinical trial of Hexavac™ in which the response rate was 87–100% for each of the antigens [3]. However, the antibody titers achieved in rhesus monkeys were ≈10-fold higher that titers attained in the human post-dose 3. The magnitude of the response of the rhesus monkeys to three doses of Hexavac™ was similar to that of human infants given four injections of Hexavac™ [3]. It should be noted, however, that the small sample siz ($n = 8$ monkeys per group) is a limitation in the interpretation of antibody titers and response rates. With these caveats, the rhesus monkey animal model described herein may be useful in the evaluation and development of future combination vaccines.

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References

[1] Ellis RW. Development of combination vaccines. Vaccine 1999;17:1635.
[2] Andre FE. Development and clinical application of new polyvalent combined paediatric vaccines. Vaccine 1999;17:1620.
[3] Pines E, Barrand M, Fabre P, Salomon H, Blondeau C, Wood SC, et al. New acellular pertussis-containing paediatric combined vaccines. Vaccine 1999;17:1650.

[4] Eskola J. Epidemiological views into possible components of paediatric combined vaccines in 2015. Biologicals 1994;22:323.
[5] Barr M, Llewellyn-Jones M. Some factors influencing the response to immunization with single and combined prophylactics. Br J Exptl Pathol 1955;36:147.
[6] Plotkin S, Koprowski H, Stokes J. Clinical trials in infants of orally administrated attenuated poliomyelitis viruses. Pediatrics 1968;23:1041.
[7] Corbel MJ. Control testing of combined vaccines: a consideration of potential problems and approaches. Biologicals 1994;22:353.
[8] Michaelis L. Weitere untersuchungen uber eiweißprazipitine. Dtsch Med Wschr 1904;30:1240.
[9] Kuo J, Monji N, Schwalbe R, McCoy D. A radioactive antigen binding assay for the measurement for the measurement of antibody to haemophilus influenzae type b capsular polysaccharide. J Immunol Methods 1981;43:35.
[10] Vella P, Staub J, Armstrong J, Dolan K, Rusk C, Szymanski S, et al. Immunogenicity of a new *Haemophilus influenzae* type b conjugate vaccine (Meningococcal protein conjugate) (Pedvax-HIB®). Pediatrics 1990;85:S668.
[11] Wang S, Liu X, Fisher K, Smith JG, Chen F, Tobery TW, et al. Enhanced type 1 immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminum-phosphate. Vaccine 2000;18:1227.
[12] Manclark C, Meade B, Burstyn D. Serological response to Bordatella pertussis. In: Rose N, Friedman H, Fahey J, editors. Manual of Clinical Laboratory Immunology, 3rd ed. Washington, DC: American Society for Microbiology, 1986:388.
[13] Bizzini B. Tetanus. In: Germanier R, editor. Bacterial Vaccines. Orlando, FL: Academic Press, 1984:37.
[14] Miyamura K, Nishio S, Ito S, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using Vero cells: I. Studies on factors affecting the toxin in microplate culture Chinese Hamster Ovary cells. J Biol Stand 1974;2:189.
[15] Gillenius P, Jaatmaa E, Askelof P, Granstrom M, Tiru M. The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese Hamster Ovary cells. J Biol Stand 1985;13:61.
[16] Fisher LD, van Belle G. Biostatistics: A Methodology for the Health Sciences. New York: Wiley, 1983.
[17] Taussig MJ. Antigenic competition. Curr Topic Microbiol Immunol 1973;60:125.
[18] Babbit BP, Matwueda G, Haber E, Unanue ER, Allen PM. Antigenic competition at the level of peptide-Ia binding. Proc Natl Acad Sci USA 1986;83:4509.
[19] Johansson BE, Moran TM, Kilbourne ED. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. Proc Natl Acad Sci USA 1987;84:6869.
[20] Pross HP, Eidinger D. Antigen competition: a review of nonspecific antigen-induced suppression. Adv Immunol 1974;18:133.

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Interference of outer membrane protein PalA with protective immunity against *Actinobacillus pleuropneumoniae* infections in vaccinated pigs

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Abstract

The role of antibodies to the outer membrane protein PalA of *Actinobacillus pleuropneumoniae* in protective immunity was studied in pigs vaccinated with purified PalA alone and PalA in combination with toxoids of the RTX toxins ApxI and ApxII using an established challenge model with the virulent serotype 1 of *A. pleuropneumoniae*. Pigs that developed antibody titers against PalA after immunization were more significantly affected by challenge with *A. pleuropneumoniae* serotype 1. Following challenge, pigs that were immunized with PalA showed more severe respiratory symptoms, had a higher mortality rate and died faster. They also displayed much more severe lung lesions after necropsy than animals not immunized with PalA. Pigs that were immunized with toxoids of the two cytotoxins ApxI and ApxII were protected against challenge with *A. pleuropneumoniae*. In contrast, the protective efficacy of the ApxI and ApxII vaccine was completely lost when it was supplemented with PalA. Hence, antibodies induced against the outer membrane protein PalA of *A. pleuropneumoniae* aggravated the consequences of infection and counteracted the protective effect of anti-ApxI and anti-ApxII antibodies. Due to the high similarity between protein analogues of PalA from various bacteria of the *Pasteurellaceae* family such as P6 of *Haemophilus influenzae* or 16 kDa Omp of *Pasteurella multocida*, this deleterious effect of PalA in vaccination should be taken into consideration in the development of vaccines against infections with other *Pasteurellaceae*.

Keywords: Outer membrane protein; Experimental infection; Antigenicity; Immunity; Vaccine; Porcine pleuropneumonia; *Pasteurellaceae*

1. Introduction

Actinobacillus pleuropneumoniae, a Gram-negative bacterium of the family *Pasteurellaceae*, is the etiological agent of porcine pleuropneumonia, a severe contagious disease of swine with worldwide prevalence [1]. To date, fifteen serotypes have been described which variously express three different cytotoxins belonging to the RTX toxin family: ApxI, ApxII, ApxIII [2]. These toxins mainly determine the virulence of the different serotypes [3]. A fourth RTX-toxin, ApxIV, which is expressed by all serotypes of *A. pleuropneumoniae*, was recently detected and shown to be produced during infection but not during culture in growth medium [4,5]. Serotypes 1, 5a/b, 9 and 11, which express ApxI, ApxII and also ApxIV, are particularly virulent, while the other serotypes which are devoid of ApxI are generally less virulent [3]. Vaccination is an effective strategy for the prevention of porcine pleuropneumonia outbreaks. Recently, a new generation subunit vaccine, composed of the three major RTX exotoxins (ApxI, ApxII and ApxIII) and a 42 kDa outer membrane protein of *A. pleuropneumoniae*, has been developed and shown to give high protection against all 12 major serotypes (serotypes 1–12) under experimental conditions [6,7] as well as in field trials [8–10]. Vaccination of pigs with the RTX toxins alone protects against mortality but generally fails to reduce the typical *A. pleuropneumoniae* lung lesions, while the combination of RTX toxins with the 42 kDa outer membrane protein (42 kDa OMP) induced complete protection with regard to mortality as well as lung lesions [6]. The 42 kDa OMP is therefore a valuable component of the vaccine. Among the OMPs of *A. pleuropneumoniae*, PalA is the most immuno-predominant antigen [11,12]. PalA is a 14 kDa protein, encoded by *palA* as a precursor peptide which is processed by signal sequence peptidase II and sorted by a peptide located signal

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for the final localizati n of the mature protein in the outer membrane. PalA is conserved in all *A. pleuropneumoniae* serotypes and induces a strong IgG response in pigs naturally or experimentally infected with *A. pleuropneumoniae* [11]. PalA shows high amino-acid (aa) sequence homology to the family of peptidoglycan associated proteins (PAL) of Gram-negative bacteria showing most similarity to the P6 protein of *Haemophilus influenzae* [11,13,14] and to the 16 kDa Omp of *Pasteurella multocida* [15]. There is evidence that PAL proteins of *Pasteurellaceae*, in particular P6 of *H. influenzae*, can act as protective antigens. P6 was therefore suggested to be included in vaccines against *H. influenzae* induced meningitis and avian cholera [15,16]. Antiserum directed against recombinant P6 protein was shown to be bactericidal against clinical *H. influenzae* isolates, including highly pathogenic non-typable strains [17]. Antibodies to P6 give passive protection to infant rats against *H. influenzae* type b-induced meningitis. In the view of the importance of PAL proteins as potential vaccine antigens, we have designed the present study to analyze the role of PalA alone, and in combination with toxoids of the RTX toxins ApxI and ApxII in induction of protective immunity against challenge of pigs with a virulent *A. pleuropneumoniae* serotype 1 strain, 4074^T.

2. Materials and methods

2.1. Bacterial strains, growth conditions and vectors

A. pleuropneumoniae 4074^T (serotype 1 reference strain) used for PCR amplification of the *palA* gene, and *A. pleuropneumoniae* serotype 1 strain 1-L-452 used for challenge, were grown on solid Columbia broth agar (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10 mM β-NAD (Sigma Chemicals, St. Louis, MO, USA) or in liquid medium consisting of Columbia broth supplemented with 10 mM β-NAD. In order to avoid appearance of contaminating bacteria during re-isolation of *A. pleuropneumoniae* from the challenged pigs, lungs and tonsils were first superficially decontaminated with a hot spatula before sampling with sterile tools from the inside of the tissues.

Escherichia coli K-12 strain XL1-blue MRF′ (Stratagene, La Jolla, CA, USA) and *E. coli* B strain BL21 (DE3) (Novagen, Madison, WI, USA) were grown in Luria-Bertani (LB) broth [18] at 37 °C in orbital shaker incubator. Ampicillin 100 μg/ml was added when needed for selection or stabilization of plasmids. Cloning vector pETHIS-1 [4] was used for the production of recombinant poly-histidine tailed peptides.

2.2. DNA extraction, manipulation, cloning and sequence analysis

Gen mic DNA from *A. pleuropneumoniae* was extracted by th guanidiumthiocyanate method [19]. Ligati n, gene cloning, plasmid extraction, restriction endonuclease digesti n and analysis of the DNA fragments by agarose gel electrophoresis were performed using standard protocols [18]. Plasmid extraction was done using the alkaline lysis method with the Miniprep kit (Qiagen AG, Basel, Switzerland). DNA sequencing reactions were performed with approximately 500 ng plasmid DNA per reaction mixture and 5 pmol of primer. Sequences were determined with an ABI Prism model 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled and edited by using the Sequencher 3.0 program (Gene Codes Corp., Ann Arbor, MI, USA) to obtain contiguous sequences.

2.3. Production of antigens for vaccines

PalA protein from *A. pleuropneumoniae* serotype 2 strain S411 for vaccination was produced as described earlier and involved heat extraction in PBS buffer (Na-phosphate 50 mM, pH 7.5, NaCl 0.85%) for 1 h at 60 °C, ammonium sulfate fractionation and separation by SDS-PAGE followed by electroelution [11]. The commercially available subunit vaccine, Porcilis App™ (Intervet International, Boxmeer, the Netherlands), was used as reference preparation for the titration of the ApxI and ApxII antigens. ApxI and ApxII proteins were prepared from supernatants of *A. pleuropneumoniae* serotype 5b bacteria grown at 37 °C for 6 h in Columbia broth supplemented with βNAD 10 μg/ml and $CaCl_2$ 25 mM. Cells were removed by centrifugation and sterile filtration through a low protein binding 0.45 μm filter (Acrocap, #4482, Gelman Laboratory, Ann Arbor, MI, USA). ApxI and ApxII were subsequently concentrated by ultrafiltration on a 500 kDa MW cut-off polyether sulfon filter (Amicon, bioseparations, Millipore, Bedford, MA, USA). Protein concentrations were measured by the method of Bradford [20]. ApxI and ApxII preparations were analyzed by standard SDS-PAGE stained with Coomassie blue [21] where they revealed a predominant band at 105 kDa indicating that ApxI and ApxII represented the major proteins (estimated to 90% of the total proteins) in the preparations [22].

For the vaccination of pigs, the following vaccines were produced: (i) Vaccine I (PalA) consisted of 80 μg PalA protein in 2 ml Diluvac Forte® adjuvant formulation (Intervet International) per vaccine dose with 0.02% (v/v) formaldehyde final concentration. (ii) Vaccine II (PalA + ApxI + ApxII) consisted of 80 μg recombinant PalA, ApxI and ApxII in 2 ml Diluvac Forte® adjuvant formulation per vaccine dose with 0.02% (v/v) formaldehyde final concentration. (iii) Vaccine III (ApxI + ApxII) consisted of ApxI and ApxII in 2 ml Diluvac Forte® adjuvant formulation per vaccine dose with 0.02% (v/v) formaldehyde final concentration; the concentrations of ApxI and ApxII in vaccines II and III were titrated by specific antigenic mass ELISA and adjusted to the same concentrations of antigens in the commercially available vaccine Porcilis App™ corresponding to approximately 50 μg of ApxI and 25 μg of ApxII per dose

(2 ml) of vaccine. (iv) Vaccine IV (Porcilis App™) was a positive protection control, using the commercially available subunit vaccine Porcilis App™ (Intervet International).

2.4. Vaccinations and challenge

Vaccination was done intramuscularly. For each vaccine, three 6 weeks old SPF landrace pigs were immunized on day 0 (zero) and subsequently received a booster vaccination on day 28. Three control animals (group V) received injections of adjutants on the same days. Two weeks after booster vaccination, on day 42, control sera were taken and then the pigs were challenged with *A. pleuropneumoniae* serotype 1 strain 1-L-452. The bacteria for challenge were grown for 6 h and washed twice by centrifugation at 10,000 × *g* for 15 min and re-suspension in the original volume of PBS buffer. Pigs of all groups were exposed at the same time for 15 min to the bacteria in an aerosol using a De Vilbiss nebulizer [23]. After challenge, fever and respiratory symptoms were recorded. Two weeks later, on day 56, the pigs were slaughtered, unless they died of the infection or had to be euthanized prior to this date. Blood was taken on the day of immunization, on day 42 (prior to challenge) and from the surviving animals at day 56. Dead and euthanized animals were subjected to post-mortem examination for typical lung lesions and recovery of challenge bacteria from lungs and tonsils.

2.5. Production of recombinant PalA'His

In order to specifically monitor the antigenic response to PalA in vaccinated pigs, we have produced an antigenically specific recombinant peptide consisting of the hydrophilic central- and C-terminal part of PalA containing the 106 most C-terminal amino acids (11.54 kDa) fused to 6 N-terminal and 10 C-terminal histidine residues. The corresponding part of the *palA* gene was amplified using the oligonucleotide primers X89009-L (cgc*catatg*-CAAACTCGTTACACCACT) and X89009-R (cgc*ggatcc*-GTATTCTAATACTGCACG). The primers were designed to contain recognition sites for the restriction enzymes *Nde*I and *Bam*HI (shown in italic letters) by the addition of supplementary nucleotides (shown in lower case). This procedure allowed the PCR amplification product to be cloned into the *Nde*I and *Bam*HI cloning sites of vector pETHIS-1. PCR was carried out with a DNA thermal cycler (GeneAmp 9600; Perkin-Elmer Cetus, Norwalk, CT, USA) in a 50 μl reaction mixture containing 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween 20, 170 μM of each dNTP, 0.25 μM forward and reverse primers, 0.5 units *Pwo* polymerase and 5 ng genomic DNA of *A. pleuropneumoniae* strain 4074. The amplification was carried out for 35 cycles (30 s denaturation at 94 °C, 30 s annealing at 50 °C, 1 min elongation at 72 °C). The PCR product was purified using the QIA quick PCR purification kit (Qiagen, Basel, Switzerland), cut with the restriction enzymes *Nde*I and *Bam*HI and ligated to *Nde*I and *Bam*HI digested pETHIS-1. Ligated DNA was transformed into *E. coli* strain XL1-blue MRF and positive clones were selected by colony hybridization using a digoxigenin labelled DNA probe for *palA* [11]. A positive clone, named pJFF-PalA1H, was sequenced to verify the integrity of the cloned segment of *palA* and the fusions with the poly-His codons of pETHIS-1. For biosynthesis of recombinant PalA'His peptide, plasmid pJFFPalA1H was introduced into *E. coli* BL21 (DE3) for expression, which was induced by addition of 1 mM IPTG at mid-exponential phase and incubation for a further 2.5 h. Following induction, the poly-histidine tailed fusion protein PalA'His was purified from cell extracts dissolved with 6 M guanidine hydrochloride using Ni^{2+} chelate affinity chromatography (Qiagen) according to the manufacturer's instructions. The bound PalA'His was eluted by slowly decreasing the pH from 8.0 to 4.5 with 50 mM potassium phosphate buffer, 300 mM NaCl, 6 M guanidine hydrochloride. Following elution at pH 4.5, the fusion protein was dialyzed against 50 mM phosphate buffer, 300 mM NaCl, pH 7.5. The fractions were analyzed by standard SDS-PAGE [18] and protein concentrations were measured by the method of Bradford [20].

2.6. Serological assays

In order to monitor the immune response to PalA in vaccinated pigs, we have developed an immunoblot assay using purified recombinant PalA'His as an antigen. Samples of 100 μg PalA'His were separated on 14% SDS-PAGE of 8 cm × 8 cm and subsequently blotted onto nitrocellulose membranes (BioRad, Hercules, CA, USA, product no. 162-0112). The dried membranes were cut into 20 strips with a width of 4 mm in order to get immunoblot strips each containing 5 μg of PalA'His. The strips were reacted with pig sera diluted 1:500, using the standard immunoblot procedure [18]. Phosphatase labelled goat antibodies, directed against pig IgG (Kirkegaard & Perry, Gaithersburg, MD, USA; product no. 051401), diluted 1:2000, followed by addition of nitroblue tetrazolium and bromochlorindolyl phosphate in alkaline phosphate buffer [18], were used to visualize bound antibodies. Each lot of immunoblot strips was controlled using rabbit anti-PalA antibodies [11] at a dilution of 1:1000 and phosphatase labelled goat antibodies directed against rabbit IgG (Kirkegaard & Perry, product no. 075-1506) diluted 1:2000.

Specific antibody titers in serum against ApxI, ApxII, ApxIII and 42 kDa OMP were determined by indirect ELISA as described [24]. This ELISA is based on the antigens ApxI, ApxII, ApxIII and 42 kDa OMP that were purified from *A. pleuropneumoniae* strains with serotypes not related to the production strains used for vaccine antigen production, in order to avoid possible cross-reaction with contaminating polysaccharides. Concentrations f antigen preparations were determined in antigenic mass ELISA (toxins) or SDS-PAGE (OMP), relativ t reference

preparations. The antigenic mass ELISA determines the concentration in arbitrary units relative t the reference preparations. The concentrations of the proteins used for coating of the micro-titer plates for antibody ELISA were in the order of 1 μg/ml. Final adjustments were made depending on antibody titers obtained with positive and negative reference sera. Routinely samples were measured twice and the mean values were reported. The differences of the two measurements were below 10%.

3. Results

3.1. Immune response to PalA in vaccinated pigs

The results of the analysis of anti-PalA antibodies in the blood sera by immunoblots with PalA'His are shown in Fig. 1. All pigs were free of anti-PalA antibodies before vaccination. Pigs vaccinated with vaccines I or II, which contained PalA alone or in combination with ApxI + ApxII, showed a weak reaction 28 days after the first vaccination, and a strong anti-PalA reaction 2 weeks after the booster vaccination. One pig belonging to group II (#176), from which a blood sample was taken before slaughter, showed an anti-PalA reaction on day 56 (Fig. 1). Groups III and IV of pigs that were vaccinated with vaccine containing ApxI + ApxII or with the commercial vaccine respectively, did not show anti-PalA antibodies after first vaccination (day 28) or after booster vaccination (day 42). One pig (#181) of group III showed a very weak anti-PalA reaction 2 weeks after challenge on day 56, which is thought to be due to the challenge with *A. pleuropneumoniae* (Fig. 1).

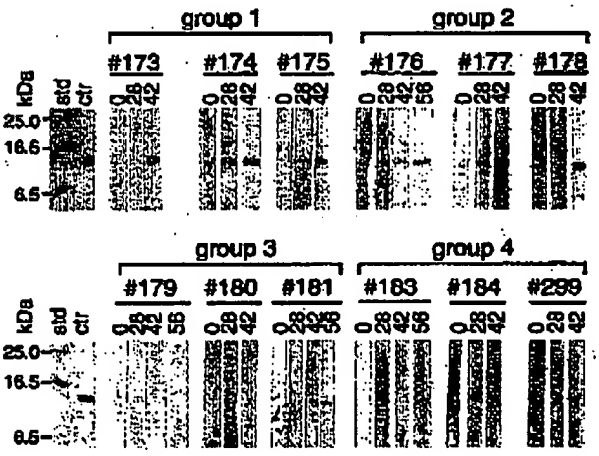

Fig. 1. Serological analysis of anti-PalA antibodies. Immunoblot strips each containing 5 μg PalA'His protein incubated with the different sera before and after vaccination are shown. Figures in the horizontal position indicate the pig numbers, figures in the vertical position indicate days after vaccination. Group I included pigs #173, #174 and #175 vaccinated with vaccine I (PalA); group II included pigs #176, #177 and #178 vaccinated with vaccine II (PalA + ApxI + ApxII); group III included pigs #179, #180 and #181 vaccinated with vaccine III (ApxI + ApxII); group IV included pigs #183, #184 and #299 vaccinated with vaccine IV (Porcilis App™, (Intervet) containing ApxI, ApxII, ApxIII and 42 kDa Omp). Note that pigs #173, #174, #175 and #177 died or were euthanised before the end of the experiment. Std: broad range pre-stained protein markers (New England Biolabs, Beverly, Mass, USA; no. 77085); the position of the molecular masses of 25, 16.5 and 6.5 kDa are indicated.

Table 1
Antibody responses after two vaccinations, measured 2 weeks after booster vaccination and before challenge

Vaccine (content)	Antibody titer against antigen			
	ApxI	ApxII	ApxIII	42 kDa OMP
I (PalA)	<2[a]	<2[a]	<2.0[a]	<2.0[a]
II (PalA + ApxI + ApxII)	3.3	2.6	<2.0[a]	<2.0[a]
III (ApxI + ApxII)	3.5	2.6	<2.0[a]	<2.0[a]
IV (Porcilis App™)	3.2	2.8	2.8	2.4
V (Control)	<2.0[a]	<2.0[a]	<2.0[a]	<2.0[a]

ELISA titers are expressed as the logarithm ($\log_{10}$) of the reciprocal of the highest dilution of serum with an OD above that of the preimmune serum for each pig diluted 1:100 as defined by [24]. Figures represent the mean values of two measurements.

[a] Indicates below detection level.

The antibody responses to vaccination against ApxI, ApxII, ApxIII and 42 kDa OMP were as expected for the various vaccines containing these antigens, as shown in Table 1. The comparison of antibody titers against ApxI and ApxII after vaccination with PalA (group II) or without PalA (group III) added, showed that the serological titers f ApxI and ApxII were not affected by the presence of PalA in the vaccine.

3.2. Protection against infection with A. pleuropneumoniae

The susceptibility of the pigs to *A. pleuropneumoniae* was assessed by challenge of a non-vaccinated group of pigs. In this group, all pigs showed high fever after the challenge as well as abdominal respiration and coughing, which are typical signs for pleuropneumonia (Table 2). One of the three pigs died 6 days post-infection. Upon necropsy, all pigs showed lung lesions affecting, on average, 50–75% of the lungs. These results showed that the challenge with *A. pleuropneumoniae* serotype 1 strain resulted in typical signs of porcine pleuropneumonia under the given experimental conditions.

The group of pigs vaccinated with the commercial vaccine Porcilis App™ (vaccine IV) showed significantly fewer clinical signs after challenge with low or no fever and virtually no respiratory distress. No mortality occurred in this group and no or only minor lung lesions affecting less than 25% of the lung were detected. These results testify to the high protection obtained with the subunit vaccine.

In the group of pigs vaccinated with ApxI and ApxII alone (vaccine III), a high level of protection against infection with *A. pleuropneumoniae* serotype 1 was observed. This was similar to the group vaccinated with the commercial subunit

Table 2
Effect of challenge with *A. pleuropneumoniae* serotype 1 on pigs vaccinated with various vaccines.

Vaccine (content)	Pig no.	Fever[a]	Respiratory distress[b]	Mortality (day p.i.)[c]	Lung lesion score[d]	Re-isolation[e]	
						Lung	Tonsil
I (PalA)	173	nd	3	+ (1)	4	+	+
	174	nd	3	+ (1)	4	+	−
	175	2	3	+ (2)	4	+	Cont.
	avg	2	3	3/3 (1.3)	4	3/3	1/2
II (PalA + ApxI + ApxII)	176	2	2	−	2	+	+
	177	0	3	+ (2)	4	Cont.	Cont.
	178	2	2	−	3	+	+
	avg	1.3	2.3	1/3 (2)	3	2/2	2/2
III (ApxI + ApxII)	179	0	0	−	0	−	+
	180	0	0	−	0	−	+
	181	1	1	−	0.5	+	−
	avg	0.3	0.3	0/3	0.2	1/3	2/3
IV (Porcilis App™)	299	1	1	−	1	+	−
	183	0	0	−	0	−	+
	184	1	0	−	1	+	−
	avg	0.7	0.3	0/3	0.6	2/3	1/3
V (Control)	185	2	2	+ (6)	4	+	+
	186	2	2	−	2	−	+
	189	2	2	−	3	+	+
	avg	2	2	1/3 (6)	3	2/3	3/3

[a] Scale used: (0) no fever (<40 °C); (1) fever (>40–41 °C); (2) high fever (>41 °C); (nd) could not be determined due to the rapid death of the animal.
[b] Scale used: (0) no distress, normal respiration; (1) increased respiration rate; (2) abdominal respiration and/or coughing; (3) dyspnea.
[c] (+) Indicates dead pig or pig euthanised because of severe clinical signs (number in brackets gives the day of death post-infection); (−) indicates that the pig did not die.
[d] Scale used: (0) no lesions; (1) 1–25% of the lungs affected; (2) 26–50% affected; (3) 51–75% affected; (4) 76–100% affected (severe lesions).
[e] (+) Indicates animal from which the challenge strain was re-isolated; Cont.: isolation of *A. pleuropneumoniae* not possible since culture was contaminated by other bacteria; (−) indicates that no *A. pleuropneumoniae* could be isolated.

vaccine. This group showed no mortality, only one pig with mild fever and increased respiration rate and virtually no lung lesions after necropsy.

Vaccine II, containing PalA added to ApxI and ApxII, showed no protection. On average, the pigs showed clinical signs similar to the non-vaccinated control group. In this group, the mortality rate was similar to that of the control group. In addition, lung lesions in this group showed the same scores as in the non-vaccinated group.

Vaccination of pigs with purified PalA alone (vaccine I) showed no protection, but rather severe symptoms and rapid death after challenge with *A. pleuropneumoniae* serotype 1. All three pigs died within 1 or 2 days after challenge and subsequent necropsy revealed severe lung lesions. In comparison with the non-vaccinated control group, vaccination of pigs with PalA alone resulted in a significant increase in mortality, three out of three PalA vaccinated animals died, compared to one animal out of three that died from the control group ($\chi^2 = 3.0$; $P = 0.08$) (Table 2). Most significant, was th sudden death after challenge of the PalA vaccinated pigs one to 2 days post-infection, while in the control group one pig died 6 days post-infection (Table 2). Upon necropsy, all pigs vaccinated with PalA showed very severe lung lesions (score 4 with 75–100% of the lungs affected) compared to one animal out of three in the control group (Table 2).

From pigs of all groups, the challenge strain could be re-isolated after necropsy from lungs and from tonsils, showing no differences between the control group and the different vaccinated groups. From a few animals, re-isolation of the challenge strain was hindered due to strong growth of other bacteria on the culture media (Table 2).

4. Discussion

The family of PAL proteins has been characterized as proteins constituting integral parts of the outer membrane of many Gram-negative bacteria. They are highly conserved within given bacterial species. Moreover, they show strong similarities between different bacterial species. They are described as very strongly antigenic proteins in several pathogenic bacteria such as *A. pleuropneumoniae* [11], *H. influenzae* [25,26], *Legionella pneumophila* [27], *P. multocida* [15], *Campylobacter jejuni* [28] and *Brucella abortus* [29]. The prototype of PAL, the peptidoglycan associated lipoprotein PAL of *E. coli* was shown to form complexes,

one type together with the outer membrane proteins TolA, TolQ and TolR, and a second type with the periplasmic protein TolB, in order to maintain the outer membrane integrity by anchoring the outer membrane to the peptidoglycan layer [30]. Mutants deficient in the PAL protein appear to be debilitated for growth under certain conditions. A PAL-deficient mutant of *Haemophilus ducrei* was shown to display a reduced capacity for pustule formation compared to its wild type parent when injected in human volunteers [31]. In addition, the mutant was more susceptible to the antibiotics Erythromycin, Cefotaxime and Ciprofloxacin than the wild type strain and could not be reisolated from pustules in contrast to the wild type strain [31]. The PAL proteins seemed therefore to be valuable targets for immune protection. Several reports of indirect evidence indicate that protein P6 (alternatively named Hi-PAL) of *H. influenzae* is involved in the induction of protective immunity against *H. influenzae* infections. They include studies showing that antibodies to P6 are protective in the infant rat model against invasive *H. influenzae* type b [32]; the demonstration of bactericidal activity for *H. influenzae* of an antibody to P6 immunopurified from human serum [33]; and the fact that rabbit antiserum raised to purified P6 is bactericidal for a broad range of *H. influenzae* strains including many non-typable *H. influenzae* [16]. From these results it was thought that P6 might be a valuable subunit in vaccines against *H. influenzae* infections. Moreover, PAL of other pathogens were considered as appropriate antigens in vaccines. However, no protection against avian cholera was btained by vaccination with recombinant P6-like protein from *P. multocida* [34].

In our approach to examine the effect of a PAL protein as candidate for a vaccine, we have taken advantage of a well established challenge model of pigs with *A. pleuropneumoniae* to test the efficacy of PalA alone or PalA in combination with known protective antigens as vaccines against porcine pleuropneumonia. In our study, a small number of animals were tested for ethical reasons and, therefore, it does not allow for thorough statistical analysis. However, our data clearly show that pigs, which developed antibody titers against PalA after immunization, showed more significant symptoms, a much higher mortality and died much faster after challenge with *A. pleuropneumoniae* than unvaccinated control pigs. The more severe lung lesions found after necropsy in the PalA vaccinated group further highlighted this observation. Hence the higher mortality and the faster occurring death in the PalA vaccinated group seemed to be an aggravation of the pleuropneumonia and was not due to secondary effects like septic shock. The difference in protective efficacy between the vaccine containing ApxI and ApxII and the vaccine with ApxI, ApxII and PalA is of particular interest. The pigs vaccinated with the two cytotoxins ApxI and ApxII were well protected against challenge with *A. pleuropneumoniae* serotype 1, like the group that was vaccinated with the commercially available subunit vaccine Porcilis App™. In contrast, the protective efficacy of ApxI and ApxII vaccine was completely lost when it was supplemented with PalA, as sh wn in the group of pigs vaccinated with vaccine II. Hence, PalA antibodies significantly reduce the protective effect of anti-ApxI and anti-ApxII antibodies. The mechanism behind this negative effect of PalA on protective immunity is not known. However, we rule out the possibility that PalA would have had a negative effect on the induction of antibodies against ApxI and ApxII, as the anti ApxI and ApxII titers are the same in the presence or absence of PalA. Since PalA is well conserved in all serotypes of *A. pleuropneumoniae*, the effect is expected to occur with any of the serotypes.

Although the limited number of animals used did not allow us to perform dose-dependence studies, we conclude that PalA should be absent in vaccines against *A. pleuropneumoniae*. Our study does not permit us to extrapolate whether other PAL antigens such as P6 of *H. influenzae* would yield similar effects. However, PalA shows very high similarity to P6 of *H. influenzae* (73% identical and 82% similar aa) and to P6-like protein of *P. multocida* (72% identical and 97% similar aa). Vaccination with other PAL proteins could therefore result in similar negative effects, or as in the case of the P6-like protein of *P. multocida*, give no protection [34]. When using whole cell preparations of bacterial cultures (bacterin vaccines), it must be noted that the concentration of PAL proteins varies depending on the mode of cultivation and preparation of the bacteria, and might therefore vary from one batch to another. Consequently, this could be an explanation of the variations in protective efficacy of certain bacterin vaccines, which are currently observed. Since the negative effect of PAL proteins in vaccines seems to be non-predictable, it would be advisable to avoid PAL proteins in vaccines unless specific evidence for a positive effect on protection is found. The existence of such negatively acting components gives further support to the need for development of well defined subunit vaccines against bacterial infections.

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References

[1] Taylor DJ. *Actinobacillus pleuropneumoniae*. In: Straw BE, D'Allaire S, Mengeling WL, Taylor DJ, editors. Diseases of swine. Ames, Iowa USA: Iowa State University Press; 1999. p. 343–54.

[2] Blacknll PJ, Klaasen HBLM, van den Bosch H, Kuhnert P, Frey J. Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet Microbiol 2002;84:47–52.

[3] Frey J. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends Microbiol 1995;3(7):257–61.

[4] Schaller A, Kuhn R, Kuhnert P, Nicolet J, Anderson TJ, MacInnes JI, et al. Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*. Microbiology 1999;145:2105–16.

[5] Schaller A, Djordjevic SP, Eamens GJ, Forbes WA, Kuhn R, Kuhnert P, et al. Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene *apxIVA*. Vet Microbiol 2001;79(1):47–62.

[6] Kobisch M, van den Bosch JF. Efficacy of an *Actinobacillus pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society Congress, The Hague, The Netherlands, vol. 12; 1992. p. 216.

[7] van den Bosch JF, Pennings AMMA, Cuijpers MECM, Pubben ANB, van Vugt FGA, van der Linden MFL. Heterologous protection induced by an *A. pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society Congress, Lausanne, Switzerland, vol. 11; 1990. p. 11.

[8] Martelli P, Guadagnini PF, Foccoli E, Ballarini G. Efficacy of an *Actinobacillus pleuropneumoniae* subunit vaccine in the control of pleuropneumonia: a field trial. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Bologna, Italy; 1996. p. 214.

[9] Pommier P, Ridremont B, Wessel-Roberts S, Keïta A. Field study into efficacy of a new *Actinobacillus pleuropneumoniae* subunit vaccine. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Bologna, Italy; 1996. p. 206.

[10] Valks MMH, Nell T, van den Bosch JF. A clinical field trial in finishing pigs to evaluate the efficacy of a new APP subunit vaccine. In: Proceedings of the International Pig Veterinary Society (IPVS) Congress, Italy; 1996. p. 208.

[11] Frey J, Kuhnert P, Villiger L, Nicolet J. Cloning and characterization of an *Actinobacillus pleuropneumoniae* outer membrane protein belonging to the family of PAL lipoproteins. Res Microbiol 1996;147(5):351–61.

[12] Rapp VJ, Ross RF. Antibody response of swine to outer membrane components of *Haemophilus pleuropneumoniae* during infection. Infect Immun 1986;54:751–60.

[13] Deich RA, Metcalf BJ, Finn CW, Farley JE, Green BA. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J Bacteriol 1988;170:489–98.

[14] Nelson MB, Apicella MA, Murphy TF, Vankeulen H, Spotila LD, Rekosh D. Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. Infect Immun 1988;56:128–34.

[15] Kasten RW, Hansen LM, Hinojoza J, Bieber D, Ruehl WW, Hirsh DC. *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *Haemophilus influenzae*. Infect Immun 1995;63:989–93.

[16] Green BA, Metcalf BJ, Quinn Dey T, Kirkley DH, Quataert SA, Deich RA. A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both nontypeable and type b *H. influenzae*. Infect Immun 1990;58:3272–8.

[17] Munson Jr RS. *Haemophilus influenzae*: surface antigens and aspects of virulence. Can J Vet Res 1990;54(Suppl):S63–7.

[18] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in molecular biology. New York, NY: Wiley; 1999.

[19] Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 1989;8:151–6.

[20] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[21] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[22] Frey J, Nicolet J. Regulation of hemolysin expression in *Actinobacillus pleuropneumoniae* serotype 1 by Ca^{2+}. Infect Immun 1988;56:2570–5.

[23] Sebunya TN, Saunders JR, Osborne AD. A model aerosol exposure system for induction of porcine *Haemophilus pleuropneumonia*. Can J Comp Med 1983;47:48–53.

[24] Nielsen R, van den Bosch JF, Plambeck T, Sorensen V, Nielsen JP. Evaluation of an indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to the Apx toxins of *Actinobacillus pleuropneumoniae*. Vet Microbiol 2000;71(1–2):81–7.

[25] Yamanaka N, Faden H. Antibody response to outer membrane protein of nontypeable *Haemophilus influenzae* in otitis-prone children. J Pediatr 1993;122:212–8.

[26] Yamanaka N, Faden H. Local antibody response to P6 of nontypable *Haemophilus influenzae* in otitis-prone and normal children. Acta Oto-Laryngol 1993;113:524–9.

[27] Engleberg NC, Howe DC, Rogers JE, Arroyo J, Eisenstein BI. Characterization of a *Legionella pneumophila* gene encoding a lipoprotein antigen. Mol Microbiol 1991;5:2021–9.

[28] Burnens A, Stucki U, Nicolet J, Frey J. Identification and characterization of an immunogenic outer membrane protein of *Campylobacter jejuni*. J Clin Microbiol 1995;33:2826–32.

[29] Tibor A, Weynants V, Denoel P, Lichtfouse B, De Bolle X, Saman E, et al. Molecular cloning, nucleotide sequence, and occurrence of a 16.5-kilodalton outer membrane protein of *Brucella abortus* with similarity to pal lipoproteins. Infect Immun 1994;62:3633–9.

[30] Bouveret E, Benedetti H, Rigal A, Loret E, Lazdunski C. In vitro characterization of peptidoglycan-associated lipoprotein (PAL)-peptidoglycan and PAL-TolB interactions. J Bacteriol 1999;181(20):6306–11.

[31] Fortney KR, Young RS, Bauer ME, Katz BP, Hood AF, Munson Jr RS, et al. Expression of peptidoglycan-associated lipoprotein is required for virulence in the human model of *Haemophilus ducreyi* infection. Infect Immun 2000;68(11):6441–8.

[32] Munson Jr RS, Granoff DM. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect Immun 1985;49(3):544–9.

[33] Murphy TF, Bartos LC, Rice PA, Nelson MB, Dudas KC, Apicella MA. Identification of a 16,600-dalton outer membrane protein on nontypeable *Haemophilus influenzae* as a target for human serum bactericidal antibody. J Clin Invest 1986;78(4):1020–7.

[34] Kasten RW, Wakenell PS, Ahmad S, Yilma TD, Hirsh DC. Lack of protection against avian cholera by vaccination with recombinant P6-like protein from *Pasteurella multocida*. Avian Dis 1997;41(4):972–6.